THE ALVEOLAR MACROPHAGE IN PULMONARY INFECTION- A COMPARISON OF PERMISSIVENESS TO INFECTION WITH AN OBLIGATE AND A FACULTATIVE INTRACELLULAR PATHOGEN

by

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ABSTRACT

The lung must mount an effective immune response to pathogenic challenge while controlling attendant tissue damage. Central to this co-ordination are the immunomodulatory effects exerted by the pulmonary environment on the local alveolar macrophages (AMs). Moreover, intracellular pathogens are known to exploit the host immune system. For this reason, we hypothesized that the resident AM would comprise a vulnerable population of cells capable of being exploited by intracellular pathogens. To test this hypothesis, we investigated the role of the AM during pulmonary infection with either the obligate intracellular bacterium *Coxiella burnetii* or the facultative intracellular fungus *Cryptococcus neoformans*.

We showed [1] that resident AM are indeed the cell-type most susceptible to infection with *C. burnetii*. These resident AM remain infected up to twelve days, serving as a permissive niche that supports bacterial survival. Additionally, a subset of infected AMs underwent a characteristic phenotypic change in response to infection, resulting in an increased expression of surface integrin CD11b and continued expression of surface integrin CD11c. We conclude that *C. burnetii* is capable of exploiting the pulmonary environmental effects on the resident AM allowing for exploitation of the AM as a susceptible and permissive niche for infection.

In the context of *C. neoformans* infection, we found that only a restricted population of *C. neoformans* replicated in AMs. The majority of *C. neoformans* that replicated *in vivo* did so extracellularly and the extent of extracellular replication exceeded intracellular replication of the yeast. In fact, the majority of intracellular fungal load in AMs at 48 hours post-infection was attributable to the uptake of extracellular *C. neoformans* from 24 to 48 hours post-infection. Thus, unlike *C. burnetii, C. neoformans* does not appear to exploit the pulmonary environmental effects on the resident AM during establishment of infection.

Through the identification of the AM as a susceptible and permissive niche for *C. burnetii* infection and the finding that *C. neoformans* replicates primarily extracellularly during early pulmonary infection, we have both filled a deficiency in the previous knowledge base and set the stage for future studies into the induction of subsequent adaptive immune responses during these varied infections.

INTRODUCTION

Lungs are specialized organs, shared in common by mammals and higher vertebrates, for the respiratory exchange of oxygen and carbon dioxide between the organism and the environment. It has been estimated that approximately 14,000 liters of air exchanges across an area of 150 m^2 daily in the human lung [2]. As a result, the lung represents a major point of contact between the internal environment of the organism and the surrounding external environment. Because of this, the lung exhibits a high susceptibility to potential insult, both environmental and pathogenic. Therefore, host defenses serving to protect against such insult at this major environmental interface are paramount.

Not surprisingly, the mammalian and particularly the human lung has evolved a complex and efficient network of physical barriers and innate immune effectors to provide defense against potential insult. These effectors have been extensively studied and are quite well understood at a general functional level. This network of effectors must be closely regulated and coordinated to not only protect from pathogenic insult, but also to avoid potentially damaging hyper-inflammatory immune responses. Therefore, as will be detailed shortly, this network represents a finely integrated system of checks and balances.

Despite broad functional understanding of pulmonary immune networks, there still remain many open questions, particularly in regard to the role of innate immune cells early in the course of pulmonary infection with intracellular pathogens. Primary among the lung innate immune cells, both due to their location and functional specialization as

phagocyte and antigen presenting cells, are the alveolar macrophages (AMs). This study, with experimental results presented in chapters 2 and 3, was designed to investigate the role of the AM in response to pulmonary infection by either the obligate intracellular bacteria *Coxiella burnetii* or the facultative intracellular yeast *Cryptococcus neoformans*. Particular attention was paid to the role of the AM in early stages of infection.

Biology of Coxiella burnetii

C. burnetii is classified as a gama-proteobacteria most closely related to the various *Legionella* species [3]. Herald Cox of Rocky Mountain National Laboratories identified and isolated the bacteria from ticks around Nine Mile Creek Montana in 1935. Concurrently, the bacterium was isolated from clinical samples in Brisbane, Australia by Edward Derrick [4]. Numerous environmental isolates have since been identified and characterized; these isolates are typically named for the geographic location of their origin [5]. *C. burnetii* is an obligate intracellular bacterium; however, cell-free growth in culture has recently been accomplished [6]. The bacteria find an environmental reservoir in various ruminants and are carried asymptomatically by these animals. The bacteria are shed to the environment in urine, feces, milk and placental tissue. From here, *C. burnetii* is able to infect humans upon aerosolization. Because this bacterium is transmitted via aerosols and in its naturally occurring form it is highly infectious, *C. burnetii* has been noted for its potential misuse as a biological weapon [7]. Clinical infection results in the disease known as Q Fever that typically presents as a self-limiting fever accompanied by flu-like symptoms and possible pneumonia. Rarely, and for still unknown reasons, *C. burnetii* infection may become chronic with severe health complications including

endocarditis [4]. The bacteria can be isolated from the environment in a stable quiescent form referred to as a small cell variant (SCV), or from the host as a metabolically active, replicative form referred to as a large cell variant (LCV) [8]. *C. burnetii* is known to infect macrophages, and IFN-γ has been implicated in resolution of the infection, whereas IL-10 has been implicated in the maintenance of infection [9]. A more detailed analysis of the interactions between *C. burnetii* and the host immune system (specifically AMs) will be addressed throughout this introduction, in the context of AM function.

Biology of Cryptococcus neoformans

C. neoformans is an encapsulated yeast and one of two recognized species in the genus *Cryptococcus*, the other species being *C. gattii* [10]. Both species are commonly found in the environment and both possess the capability to infect humans [11]. In general, the designation *Cryptococcus* may refer to a number of strains and serotypes of either of these two species, with naming conventions having changed several times over the past century [12]. More specifically, *Cryptococcus* has been referred to as a species complex encompassing both species, the various serotypes of each species, and all of the strains of each species in either of the two known forms of this dimorphic fungus (filamentous or yeast). Most often, it is the yeast form that is observed in the context of cryptococcal infection and therefore, it is this form which is referred to here-after [13]. *C. neoformans* is typically thought of as an opportunistic pathogen and is of particular concern in immunocompromised individuals; annual new cases world-wide have been estimated at nearly one million, with resultant deaths from disseminated fungal

meningitis estimated at over a half-million annually [14]. *C. neoformans* is known to infect macrophages and has been classified as a facultative intracellular pathogen after the observation that the yeast is not only able to survive, but also replicate within macrophages [15,16]. Persistent infection has been linked to intracellular survival within the host [17] and innate immune function appears to be important in the control of infection [18]. Moreover, the yeast is thought to exploit the macrophage as a vehicle for systemic dissemination [19]. A more detailed analysis of the interactions between *C. neoformans* and the AMs will be addressed throughout this introduction, in the context of AM function.

Pulmonary Innate Immune Systems

Within pulmonary airways, mucus is secreted by Goblet cells present in the airway epithelium [20]. Mucus serves as a physical barrier in the lung, functioning to trap inhaled antigens which are then physically removed from the airway by the motion of epithelial cillia, as part of the mucociliary escalator [21]. The importance of this system in barrier protection is notable in cases of its dysfunction, such as during cystic fibrosis (CF), which is marked by increased incidence of pulmonary bacterial infection [22]. However, as with many pulmonary responses, the cause and effect relationship is quite complex. It has been shown that not only is increased mucus production and decreased ciliary response a predisposing factor to an increased incidence of bacterial infection during CF, so too is reduced pulmonary lymphocyte function and increased pulmonary epithelium expression of cell surface receptors specific to the infecting bacterial species

[23]. Both of these changes highlight the highly interconnected nature of pulmonary innate immune systems.

Beyond the physical barrier function of secreted mucus within the conducting upper airway, the deep respiratory airway surfaces of the lung are bathed in alveolar lining material (ALM), containing several types of proteins and surfactants involved both in the protection and function of the lung [24]. The surfactants are produced by type II epithelial cells (type I epithelial cells function primarily in air exchange) [2]. ALM contains four different surfactant proteins of two major forms. Surfactant proteins B and C function to aid in alveolar competency and air exchange, whereas surfactant proteins A and D contain collectins participating in antimicrobial defense and innate immune regulation [2]. Although surfactant proteins A and D possess broadly anitmicrobial function, susceptibility to surfactant-mediated defenses appears to be pathogen specific, as *C. neoformans* is not susceptible to SP-A mediated clearance [25].

Interestingly, surfactant proteins A and D can function either in the promotion of pulmonary immunosuppression or pro-inflammatory activation of AMs [26]. It was shown by Gardai et. al. [26], that these proteins contain head and tail regions. The head of the proteins serve as pattern recognition receptors (PRR) and recognize exogenous antigen, binding pathogen associated molecular patterns (PAMPs). The tail regions interact with AMs and other resident phagocytes to promote phagocytic uptake of bound PAMPs. In this paradigm, tail binding occurs through the calreticulin/CD91 complex on the resident phagocyte and results in activation of the phagocyte. However, in the absence of PAMPs, the surfactant protein head interacts with the membrane bound

inhibitory receptor SIRP-α on the phagocyte, resulting in suppression of phagocyte activity. Moreover, application of surfactant phospholipids lacking either protein A or protein D has been shown to suppress nitric oxide (NO) production and NF-κB signaling in cultured AMs, suggesting that it is not exclusively the surfactant protein which plays a role in regulating the immune status of the lung [27].

Surfactant protein A (SP-A) has further been shown to upregulate surface expression of toll-like receptor 2 (TLR2) on AMs; however, SP-A also abrogates signaling through TLR2 and TLR4 resulting in a reduced production of inflammatory cytokines [28]. Henning et. al. [28], suggest that this dichotomous role of SP-A allows for an enhanced recognition of PAMPs while controlling deleterious excessive pro-inflammatory responses. This is a specific example of the general need of the lungs to tightly regulate immune response in necessity to protect against unnecessary host tissue damage. This coordinated immune response allows for adequate defense against immune challenge while controlling potentially harmful pro-inflammatory immune responses.

Both surfactant proteins A and D are degraded and recycled by AMs [29,30]. It has been suggested that, during inflammation, there is both a decrease in the rate of surfactant protein A and protein D production, as well as an increase in the rate of surfactant protein recycling [31]. Thus, whereas surfactant proteins function both as innate immune effectors and immune regulators, AMs share these functional roles and coordinate closely with any changes taking place within the pulmonary environment. In fact, it has been shown that pulmonary immunosuppression by AMs can partially protect from the detrimental effects of type II epithelial cell depletion [32]. This supports the idea

that the lung presents a tightly coordinated, non-redundant, network of immune mechanisms that both defend the lung from pathogenic insult and protect it from inflammatory damage.

In addition to the production of mucus in the upper airways and surfactants in the alveoli, pulmonary epithelial cells are known to directly influence the pulmonary environment that in turn, regulates AM phenotype and function. It has been shown that yet to be identified soluble factors secreted from pulmonary epithelial cells possess both immunoregulatory and bacteriostatic capabilities [33]. Alveolar epithelial cells also express integrin $\alpha_v\beta_6$, which is involved in TGF-β activation and inflammation control within the lung [34,35,36]. Tracheal and pulmonary epithelial cells have additionally been shown to produce GM-CSF [37,38,39]. GM-CSF has been implicated in mediating both the maintenance of AM populations [40], as well as the phenotype of AMs [41]. Additionally, alveolar epithelial cells produce IL-10, which, via binding to the IL-10 receptor (IL-10R) on AMs, results in AM immunosuppression and decreased production of inflammatory cytokines such as, TNF-α, IL-1 and IL-6 [42]. IL-10 is also capable of downregulating macrophage function as an antigen presenting cell (APC) through a decrease in expression levels of MHC-II and CD40 [43].

The alveolus has been shown to contain additional immune effectors including acute phase reactants and complement [44] produced, in part, by pulmonary epithelium. Acute phase reactants, ApoE and transferrin, have been shown to be produced by type I alveolar epithelial cells [45]. This novel functional role for the type I cells is thought to protect the lung from oxidative insult. Complement proteins, including C2, C3, C4 and

C5 have been shown to be produced by type II alveolar epithelial cells [46]. The alveolar epithelium serves as a regulator of immune function and directly aids in the protection of the lung from both exogenous insult and overly robust inflammatory responses.

In another role as mediator of immunoregulation via a direct physical contact, alveolar epithelial cells express CD200 molecules on their cell surface. CD200 is the ligand for CD200R (the receptor molecule highly expressed on the cell surface of AMs) and this ligand-receptor interaction results in AM immunosuppression [47]. Although the direct mechanism of this immunosuppression is not known, it has been speculated that CD200R signaling is mediated through SHIP [47]. SHIP is an acronym standing for SH2-domain containing inositol-5-phosphatase, a molecule involved in numerous signaling pathways including the downregulation of TNF-α and IL-6 production through the inhibition of NF-κB [48]. Alveolar epithelial cells also secrete beta-defensins, possessing anti-microbial capabilities [49]. Thus, alveolar epithelial cells not only play a role in surfactant production and mechanical function of the lung, they secrete additional effector proteins and cytokines directly affecting AM function and subsequent pulmonary innate immune response.

AM Biology

As noted earlier, at the nexus of the pulmonary defense network is the AM. During bacterial challenge, the AM is able to clear bacteria via a number of innate mechanisms and in cases where these mechanisms are insufficient for resolution of the bacterial challenge, AMs are instrumental in the recruitment of other immune effector

cells, including neutrophils, monocytes and T cells [50]. Because of the capacity to induce inflammation in the lungs, the AM activation state is tightly regulated. With this tight regulation and its function as a professional phagocyte, the AM has been recognized as a primary and pivotal pulmonary innate immune cell [51]. The role of phagocytic clearance of bacteria by the AM has long been recognized [52], as has AM's function as an APC [53]. Indeed, the central location at the air-epithelial interface, along with the phagocytic capacity of AM, was realized by electron microscopy studies more than five decades ago [54,55].

Nearly forty-five years ago, the origin of macrophages was delineated in what has come to be called the mononuclear phagocyte system [56]. In this system, it was claimed that a bone marrow (BM) precursor of monocytes, termed the premonocyte, gave rise to circulating monocytes. These circulating monocytes, in turn, exited circulation and entered tissue, subsequently differentiating into tissue resident macrophages [57,58]. Over a decade ago, this system began to be called into question [56]. At the time, Hume pointed to new experimental findings, which supported the presence of a distinctly differentiated phagocyte cell identifiable during embryogenesis [59]. Other studies showed a pulmonary, not a hematopoietic, origin for at least some AM populations [60]. Moreover, Hume argued that phenotypic and gene expression profiling results were not consistent with terminal macrophage differentiation and supported the context dependent expression of characteristic genes and phenotypic markers [61,62]. Finally, he pointed to findings that supported the role for tissue resident macrophage self-renewal when questioning the accuracy of the accepted mononuclear phagocyte system [63]. In the

alveolar compartment, the embryonic origin of tissue resident AMs has recently been confirmed [64]. Moreover, it has recently been shown that AMs possess the ability to self-renew under steady state conditions [40], as well as in the context of Th2 driven inflammation [65]. Thus, current research supports the concept of the AM as a specific type of tissue integrated macrophage, distinct from monocytes or tissue resident macrophages residing in other tissues. In fact this distinction has been supported by proteome analysis that has revealed a distinct AM protein expression profile [66]. However, numerous studies support the widely accepted view that macrophage heterogeneity increases drastically during inflammation, as circulating monocytes exhibiting widely varied and often pathogen-dependent activation states are recruited to the lung [67,68,69,70,71,72,73]. In particular, $Gr1^{+}$ monocytes have been referred to as inflammatory monocytes [74], and it has been shown that these inflammatory monocytes do not possess the ability to directly differentiate into resident AM [75]. While AM heterogeneity increases during inflammation, recruited cells do not appear to maintain long-term residency in the lung.

With respect to defining phenotypic markers, AM surface expression of IgG Fc region receptors and complement receptors has been noted and these receptors play a functional role in facilitating AM mediated phagocytosis [76]. There is a high degree of cross-talk between the AM and the surrounding pulmonary environment. Notably, AM posses surface receptors for both SP-A and SP-D [77,78]. In fact, in addition to binding a cognate receptor, SP-A is able to bind CR3 (the CD11b/CD18 heterodimer), one of two defining surface expressed complement receptors present on AM (CR3 and CR4 (the

CD11c/CD18 heterodimer)), and alter the expression level of CR3 [79]. As will be discussed further, a recent study suggests that CD11b and CD11c AM surface expression provide greater discriminatory power for heterogeneous AM populations than do typical markers of classical (M1) macrophage activation and alternative (M2) macrophage activation [80].

CR3 has been implicated as an important cell surface receptor involved in phagocytosis of both *C. burnetii* and *C. neoformans*. It has been suggested that CR3 serves as one of the cell surface receptors that facilitates phagocytosis upon interaction with *C. burnetii* [81]. Interestingly, CR3 is also a key mediator of *C. neoformans* phagocytosis. However, this CR3-initiated phagocytosis is mediated by antibodies and not by complement [82]. It was found that surface binding of either IgA or IgM to the capsule of *C. neoformans* facilitated direct interaction of the capsule and the CR3 host cell receptor, independent of complement.

AMs also express several of the c-type lectin superfamily of receptors on their cell surface. Notable among these receptors are Dectin-1 and the mannose receptor (CD206) [83]. As noted above, GM-CSF plays a role in determining AM phenotype and Serenzai et. al. [83] showed that GM-CSF controls the levels of Dectin-1 surface expression. Despite its role as the surface receptor for beta-glucan, a fungal cell wall component, Dectin-1 is not essential in innate immune responses to *C. neoformans* infection [84]. *C. burnetii* infection has been shown to result in the upregulation of mannose receptor expression (mannose receptor is a marker of M2 activation and *C.*

burnetii infection skews macrophage response toward M2 activation, as will be discussed in detail in the next section of the introduction) [85].

Cell surface and intracellular expression of TLRs by AM is common and TLR2, TLR4, and TLR9 expression levels are of particular note. Engagement of these TLRs has been shown to downregulate IL-10R function on AMs, allowing for a scalable means of AM activation [42]. In fact, it has been proposed that a contributing factor leading to increased susceptibility to bacterial pneumonia following influenza infection has to do with the sustained desensitization of TLR responses in the lung upon resolution of influenza [86]. As was mentioned earlier, SP-A interaction with the AM not only upregulates TLR2 expression, but also regulates TLR2-mediated signaling. This is of functional importance during pulmonary infection, as it has been shown that LPS stimulation of AM increases IL-1β and IL-6 production and maintains p-38 MAPK in a phosphorylated state (all indicators of AM priming and activation) [87]. As a result of this maintained activation AM, uniquely among macrophages, do not become tolerized to LPS stimulation. As inflammation begins to resolve and levels of secreted SP-A rise, the resultant downregulation of TLR2 signaling could serve to counterbalance the continued activation of AMs. In another example of the interplay between the pulmonary environment and the AMs, this lack of tolerance induction to LPS stimulation has been further tied to the effects of GM-CSF expression (along with IL-18 and IFN-γ production) on AM activation [88].

TLR2 is important in mediating host response to *C. burnetii* infection, with $TLR2^{-/-}$ animals exhibiting lower levels of IL-12 and TNF-α production along with

reduced granuloma formation compared to wild-type animals [89,90]. However, TLR2$^{-/-}$ animals were shown in these studies to be able to ultimately clear the bacteria. This is consistent with findings from our lab indicating that whereas the innate immune response is important for setting up a productive adaptive T cell-mediated responses to *C. burnetii* infection, it is ultimately the T cell response that is required for bacterial clearance [91]. This is further consistent with recent work showing protective immunization against *C. burnetii* resulted from the induction of a Th1 adaptive immune response, implicating the role of Th1 mediated immunity in the resolution of infection [92].

Along with the role of CR3 and TLR2 in mediating *C. burnetii* phagocytosis, TLR4 has been implicated as well [93]. *C. burnetii* LPS was shown to interact with TLR4 resulting in both increased phagocytosis, as well as, upregulation of both TNF-α and IFN-γ production. TLR4 signaling, in this context, leads directly to an increase in host pro-inflammatory responses along with a subsequent increase in host granuloma formation in the control of *C. burnetii* infection.

Numerous studies have elucidated the role of TLR9 in *C. neoformans* infection [94,95,96,97,98,99,100]. Specifically, in the absence of TLR9 signaling, AM activation is skewed to an M2 response (AM activation states will be discussed in detail in the next section) with impaired T cell recruitment [96]. Similarly, T cell activation and IFN-γ production during *C. neoformans* infection have been shown to be TLR9-dependent [100]. Finally, additional exogenous stimulation of TLR9 in a murine model of *C. neoformans* infection results in a Th1 adaptive immune response [94]. This is also

consistent with the observation that resolution of *C. neoformans* infection is dependent on a Th1-mediated adaptive immune response to infection [101].

In addition to specialized surface receptors facilitating complement-mediated phagocytosis and antibody-mediated phagocytosis, AMs also express surface receptors facilitating non-opsonized phagocytosis [102]. These receptors not only recognize LPS and bacterial cell wall components, they also recognize foreign exogenous environmental particles, such as dust. These receptors constitute a broad family of receptors referred to as scavenger receptors (SRs). Most notable among this family is the SR MARCO [103]. Interestingly, in another example of the tightly coordinated control of pulmonary immune responses, MARCO has been shown to have functions beyond facilitating phagocytosis of potentially damaging exogenous particles. MARCO-dependent phagocytosis of oxidized lipids, following the induction of pulmonary inflammation, has been shown to serve an important role in the protection of lung tissue from inflammatory injury [104]. While SRs function primarily in facilitating the phagocytosis of non-opsonized particles, it has been shown that they possess the ability to function as TLR2 co-receptors in the clearance of *C. neoformans* from the murine lung [105].

AM Phenotypes

As a defining physical characteristic, AMs are remarkable for high levels of autofluorescence. In fact, this autofluorescence has been used for the separation of AM from pulmonary dendritic cell (DC) populations in fluorescence-activated cell sorting (FACS) analysis [106]. It has been suggested that this unique physical characteristic of AM is a result of intracellular concentrations of flavoproteins present in AMs [107].

Functionally, this is quite consistent with the role of NADPH-oxidase and the robust oxidative burst observed in AMs. Interestingly, research suggests that not only does this oxidative burst have the anticipated anti-microbial function, but it could also serve as a form of signal transduction initiating downstream pro-inflammatory cytokine activation [108,109].

With respect to the surface expression of various cellular receptors and the general phenotype of AMs, it was noted nearly two decades ago that non-activated AM isolated from human subjects displayed AM-specific phenotypic profiles not shared by either circulating monocytes or neutrophils isolated from the same subjects [110,111]. In particular, AMs express high levels of FcγR1 (the high affinity IgG receptor [112]) and HLA-DR (human MHC-II [113]). Expression of CR4 (CD11c/CD18) was also noted; however, very low levels of CR3 (CD11b/CD18) were noted in resting AM compared to monocytes. A recently published study is the latest to undertake a more comprehensive FACS analysis of phenotypic profiling on various pulmonary monocyte, macrophage, and DC subsets [114]. Based on this phenotypic analysis, the authors define AMs as Siglec F^+ $CD11b^-$ $CD11c^+$ $CD64^+$. This is in contrast to DCs, which are defined in two populations as either $CD11c^+$ $CD103^+$ $CD24^+$, or $CD11b^+$ MHC-II^+ $CD11c^+$ $CD24^+$ $CD64^-$, and undifferentiated monocytes, defined as $CD11b^+$ MHC-II^- $CD64^{+/-}$ $Ly6C^{lo}$. As an indication of the ongoing debate and evolution within the field of pulmonary APC phenotyping, another group has published a paper phenotyping alveolar myeloid cell subsets in the mouse in the same journal and issue as the one referenced above. This group defines AM as $CD45^+$/$CD68^{hi}$/$F4/80^+$/$CD11b^-$/$CD11c^+$/$Gr1^-$, DCs as

$CD45^{+}/CD68^{hi}/F4/80^{-}/CD11c^{+}/Gr1^{-}/CD103^{+}/MHC^{-}II^{hi}$, interstitial macrophages as $CD45^{+}/CD68^{low}/F4/80^{+}/CD11b^{+}/CD11c^{+}/Gr1^{-}/CD14^{low}$, and monocytes as $CD45^{+}/CD68^{low}/F4/80^{+}/CD11b^{+}/CD11c^{-}/Gr1^{low}/CD14^{hi}$[115]. Additional studies have shown that the type of activating stimulus applied to AMs has a direct effect on the resulting phenotype. Specifically, IFN-γ activation was shown to induce CD64 expression, whereas IL-4 activation was shown to induce both CD200 and mannose receptor expression [116]. This not only highlights the observation that "conventional" naming schemes for subsets of alveolar APCs are context-dependent and may be misleading without accompanying phenotypic definitions, but it further marks some shared commonality in separating an AM from a monocyte. Specifically, the steady-state AM is recognized as $CD11c^{+}/CD11b^{-}$, while the monocyte is recognized as $CD11c^{-}/CD11b^{+}$.

As mentioned earlier, this phenotypic profiling, based on CD11c and CD11b expression, allows for the discrimination of three distinct pulmonary macrophage populations present in either a steady-state lung or in the context of LPS-induced inflammation [80]. Here, a tissue resident population of AMs was identified as $CD11c^{high}CD11b^{neg}$. During inflammation, a subset of this population was shown to upregulate CD11b expression. Further, it was shown that subsequent to this phenotypic change on a subset of resident AMs, $CD11c^{neg}CD11b^{+}$ monocytes were recruited to the lung. It was established that these cells exhibited a mixed M1/M2 phenotype characterized by both iNOS expression and Arginase-1 expression. The authors also showed that upon inflammatory resolution, the AMs return to a homogenous population

of $CD11c^{high}CD11b^{neg}$ cells. In a model of chronic lung disease, in $SHIP^{-/-}$ mice (SHIP was mentioned above and is also known to mediate increased IL-4 production resulting in M2 macrophage activation [117]), a maintained population of $CD11c^{high}CD11b^{pos}$ resident AM was observed during the course of lung disease [80].

In chapter 2 of this dissertation, we present results consistent with the observation of Duan et. al. [80], that a subset of tissue resident AMs upregulate CD11b expression during inflammatory insult. We show that in the context of *C. burnetii* infection, a subset of tissue resident AM upregulate CD11b expression, and it is this subset, which is most susceptible to infection. Thus, within the heterogeneous population of pulmonary macrophages present during infection or inflammation, it is possible to trace three distinct populations. In both the work of Duan et. al. [80], and our work presented in chapter 2, it is possible to observe a tissue resident AM population expressing a $CD11c^{high}CD11b^{neg}$ phenotypic profile in the steady-state lung. Subsequent to inflammatory or pathogenic insult, a subset of this population begins to express a $CD11c^{high}CD11b^{pos}$ profile. Shortly after this, recruited monocytes enter the lung exhibiting a $CD11c^{neg}CD11b^{high}$ expression profile and begin to manifest a $CD11c^{pos}CD11b^{pos}$ profile. As shown by Duan et. al. [80], these distinct phenotypic population profiles also have functional relevance. As we show in chapter 2, these subsets have varying susceptibility to certain pathogenic insults, consistent with functional differences in these subsets. Further, as was introduced previously, the effects of GM-CSF appear to be central in determining this pulmonary specific $CD11c^{+}$ AM phenotype. When, peritoneal macrophages (normally $CD11c^{-}$) were transferred to the lungs of recipient mice, CD11c was upregulated; however, this

occurred in a GM-CSF dependent fashion, as GM-CSF $^{-/-}$ mice did not exhibit a similar CD11c upregulation upon transfer of peritoneal macrophages into the lung [41].

Classically and Alternatively Activated AM

Within the field of macrophage biology, much current focus is being directed toward understanding differing types of macrophage activation. Central to this, a distinction between classically activated macrophages (M1) and alternatively activated macrophages (M2) is being recognized (with the naming conventions being borrowed from the Th1/Th2 distinctions of T cell biology [118]). M1 activation has been shown to follow macrophage stimulation with LPS or IFN-γ, resulting in an upregulation of FcγRIII (the low affinity IgG receptor) and iNOS, along with pro-inflammatory cytokine production (particularly IL-12, TNF-α and IL-6) and a subsequent induction of Th1-mediated adaptive immune responses [119]. M2, or alternative activation, has been further sub-divided into three distinct activations states (M2a, M2b, M2c) [119]. However, in general, M2 activation results from IL-4, IL-10 and IL-13 stimulation and leads to increased arginase-1 and mannose receptor expression, along with increased IL-10 and TGF-β production by macrophages. There is a subsequent induction of Th2-mediated adaptive immune responses and an increase in immunoregulation and tissue remodeling under these conditions [119].

Interestingly, while significant functional overlap between human and murine macrophage activation states is predicted [120], certain species specific phenotypic differences between activation states has been noted. In particular, IL-4 induced gene

expression profiles have been observed to vary between humans and mice [120]. Human CD206 (mannose receptor) expression is not varied in response to IL-4, as it is in mice [121]. Thus, increases in mannose receptor expression levels only serves as a marker for alternative macrophage activation in mice. However, CD80 and CD200R expression levels increase considerably on alternatively activated human macrophages [121]. Likewise, arginase-1 expression is considered to be a murine specific phenotypic marker of alternative macrophage activation [122]. These species specific phenotypic expression differences notwithstanding, M1 macrophages have been implicated in the abrogation of wound healing during inflammation in both humans and mice [123]. This is a specific example supporting a functional overlap between macrophage activation states in both humans and mice.

As has been discussed above, pulmonary TGF-β activation is, in part, mediated through alveolar epithelial cell expression of integrin $\alpha_v\beta_6$. The direct effect of pulmonary TGF-β on AM phenotype and activation has been shown in TGF-β receptor conditional knock-out mice. These mice exhibit M1 skewing with spontaneous inflammation in the lung [124]. This is yet another example of the effects of the pulmonary milieu on homeostasis and pulmonary innate immune function.

Interestingly, the role of AM beyond being effectors of innate immunity is beginning to be realized. A recent study found that M2 polarization of AM during development in the postnatal lungs of mice leads to an increase in AM numbers, as well as, an increased concentration of AMs at points of developing pulmonary bifurcation

[125]. This is consistent with the identified role of M2 macrophages in tissue remodeling and suggests the possibility of AM function beyond the maintenance of lung homeostasis.

Although phenotyping based on CD11c and CD11b expression levels has functional relevance in the pulmonary environment, the distinction between classical activation (M1) and alternative activation (M2) has also been studied in the lung. It has been shown that both chemical pulmonary insult with butylated hydroxytoluene (BHT) and bacterial pulmonary insult with tuberculosis (TB) resulted in a biphasic response where increased levels of IFN-γ in the lung drove AM polarization and activation toward an M1 state [126]. Upon resolution of this inflammatory response, IL-4 levels increased and AMs were driven to an M2 state. Interestingly, AMs involved in granuloma formation in the TB model exhibited no inflammatory resolution and movement away from the M1 activation state. This differential AM polarization has also been shown to be important in response to both *C. burnetii* and *C. neoformans* infection. In the first instance, *C. burnetii* has been shown to drive an unproductive M2 response in AMs within the context of pulmonary infection [85]. In the second instance, laccase (laccase is an enzyme that is recognized as a *C. neoformans* virulence factor because of its role in protective iron oxidase function [127], and melanin synthesis [128]) production by *C. neoformans* has been implicated in the induction of an unproductive M2 polarization of AMs [129].

To prevent tissue damage and lung dysfunction, subsequent to AM activation and pulmonary inflammation, the pulmonary environment must effect coordinated resolution. It is also important that the source of the original inflammation and AM activation is

effectively dealt with prior to the resolution of the immune response. This resolution phase of pulmonary immunoregulation is often accomplished through similar responses as were induced in the maintenance of pulmonary homeostasis. However, additional effects of inflammatory resolution have been noted, such as, prolonged and sustained TLR hyporesponsiveness on AMs [47]. In fact, here the authors point to susceptibility to secondary infection following resolution of pulmonary hyper-inflammation as an indication that resolution of inflammation does not simply reset pulmonary immune function, but instead it alters immune function with profound functional ramifications. The interconnected and tightly regulated pulmonary immune environment can be directly responsible for feed-forward changes that modulate future pulmonary immune responses.

AMs are remarkable for being a long-lived and stable cellular population within the alveolus, with a half-life in the steady-state murine lung being estimated at greater than four months and possibly up to one year [71,130]. Interestingly, this increased AM life-span has been purported to be promoted through SP-A signaling leading to the activation of the cell survival mediators ERK and Akt [26,131,132,133]. Importantly, during the resolution of pulmonary inflammation, it has also been determined that the activated resident AMs undergo immunosupression and not programmed cell death (apoptosis) with subsequent cellular replacement [134]. Here, the authors show that only recruited monocytes entering the lung in response to the induction of inflammation are subject to Fas-mediated apoptosis during the resolution of inflammation. Moreover, it is the resident AM that is implicated in clearance of these apoptotic cells [135]. The observation that it is the resident AM surviving and mediating “clean-up” after

inflammation has important functional implications for continued AM susceptibility to infection as the AM enters homeostasis following inflammation. In particular, with respect to the studies presented in chapters 2 and 3 of this dissertation, if intracellular pathogens are capable of exploiting the AMs as a permissive niche for infection, and thus surviving the first wave of pulmonary inflammation, it may be possible to persist in a chronic infective state within the permissive AM niche. Consistent with this possibility is the observation that many intracellular pathogens inhibit macrophage production of pro-inflammatory cytokines [136,137]. Indeed, the findings presented in chapter 2 support the existence of such a permissive AM niche involved in the maintenance of *C. burnetii* infection. In contrast, the findings presented in chapter 3 support the avoidance of AM interaction as the principle infective strategy of *C. neoformans*.

Role of AM in Resistance to Bacteria

Despite the myriad network of host defenses, the lung is still susceptible to pathogenic insult. Both extracellular and intracellular pathogens are capable of initiating and maintaining pulmonary infection within the host, with the possibility of subsequent systemic infection. As has been noted, dogma tends to assign a greater role for antibody-mediated immunity to the clearance of extracellular pathogens and a greater role to cellular (and by extension innate) immunity to the clearance of intracellular pathogens [138]. However, as Casadevall points out, this distinction is somewhat contrived, particularly with respect to facultative intracellular pathogens. However, it is still interesting to note that many highly infectious pathogens survive by immune subversion, while many less infectious pathogens survive by rapid replication [139]. This suggests

that, in certain infection scenarios, low doses of intracellular pathogens may be able to exploit the limited population of resident AMs as a niche for survival and persistence.

AMs, as do other macrophages, possess numerous methods of microbial killing. One of the primary methods of microbial killing involves reactive oxygen species (ROS) produced through a membrane bound NADPH-oxidase catalyzed reaction referred to as the oxidative burst (and mentioned previously when discussing the sources of AM autofluorescence) [140]. Again, because tight control of pulmonary immune responses is critical, AM activation and subsequent TNF-α signaling leading to an increase in ROS production is countered by an increase in cellular levels of superoxide dismutase (SOD). SOD functions to reduce ROS concentrations in the activated AMs, but it also increases TNF-α signaling and further enhances AM activation [141]. SOD has also been shown to induce expression of inducible nitric-oxide synthase (iNOS), leading to an increase in cellular concentrations of NO [142]. NO is capable of controlling a wide array of microbial pathogenic insult, including providing partial control for *C. burnetii* infection [143,144,145]. Additionally, AM-produced NO has been shown to interact with T cell signaling pathways blocking cellular proliferation [146]. Further, the interaction of superoxide anion (a ROS) with SOD results in the formation of hydrogen peroxide, which has antimicrobial properties of its own. The interaction of NO and superoxide anion can also lead to the formation of the microbicidal peroxynitrite [140]. An interconnected network of reactive oxygen and nitrogen species, along with associated by-products, is employed by macrophages as an antimicrobial defense and plays a role in the control of adaptive immune responses.

When macrophages phagocytose exogenous material, including pathogens and apoptotic cellular debris, intracellular uptake is facilitated through the formation of a phagosome. These membrane bound vacuoles are formed by inclusions of the cellular membrane and thus, initially, bear a high degree of structural similarity to the cellular plasma membrane [147]. The phagosome very rapidly acquires markers characteristic of endosomal cellular trafficking and subsequent to this, the phagosome undergoes lysosomal fusion [148]. Lysosomal fusion leads to acidic and proteolytic degradation of engulfed material, along with subsequent antigen presentation through the canonical MHC-II presentation pathway, or through the non-canonical MHC-I cross-presentation pathway [149]. Phagosomes may also gain markers signaling commitment to the autophagy pathway of cell death, as has been suggested for *C. burnetii* containing vacuoles [150]. More recently, a role for phagosomes beyond that of killing has been recognized. In particular, phagosomes are being seen as playing a role in further coordinating innate immune responses through enhancement of TLR signaling, both from the recognized intracellular TLRs and also from phagosome bound "surface" TLRs [151].

Interestingly, the role of the phagosome in sequestering engulfed bacteria from required nutrients is beginning to be appreciated. Specific examples of transporters and enzymes contained within the phagosome that limit bacterial access to required nutrients and co-factors include the NRAMP transporter and the enzyme indolamine 2,3-dioxygenase (IDO) [152]. As described by Appleburg [152], NRAMP is involved in iron transport and its presence within the phagosomal membrane can serve to limit iron availability to bacteria. It has been noted that this particular transporter is important in

response to *Leshmania* infection and infection by various *Mycobacterium* species [153,154]. IDO is involved in tryptophan metabolism [152] and its function has been shown to be important in fighting both infection with *Chlamydia* and *Toxoplasma* [155]. The phagosome plays a key role in pathogen killing and degradation, antigen presentation and pathogen sequestration.

Lastly, immune defense mechanisms of the inflammasome represent an area of intense ongoing research. In general, the inflammasome is considered to be any one of several specific cytosolic protein platforms involved in the induction of inflammation through caspase activation. These inflammasomes may be activated by various PRR signaling pathways, including TLR signaling, cytosolic Nod-like receptor (NLR) signaling, and RIG-I signaling [156]. Regardless of the initiating signaling events, or the particular inflammasome activated, Bauernfeind et. al. [156] note that the end result is IL1-mediated pro-inflammatory cytokine production and pyroptosis (inflammatory cell death). This is yet another antimicrobial strategy employed by phagocytes in general and AMs in particular.

Pathogen Strategies to Counter Host Immune Responses

A simple counter mechanism to AM-mediated pathogen killing employed by *C. neoformans*, along with diverse other pulmonary pathogens, is the avoidance of phagocytosis. For *C. neoformans* this is accomplished primarily through the protection of a polysaccharide coating, as well as the secretion of proteins serving to disrupt phagocytosis [157]. In this way, *C. neoformans* is able to avoid AM interaction and potential AM-mediated killing. Similarly, a study by Jonsson et. al. [158] found a

correlation between the pathogenicity of *S. pneumoniae* and *H. influenzae* and the ability to resist phagocytosis by host effector cells. In contrast, the authors found that the pathogenicity of *S. aureus* correlated to its ability to avoid killing by host AMs. Thus in lieu of avoiding phagocytosis, the ability to alter or persist through host immune response becomes critical.

A comprehensive review by Schmid-Hempel outlined a number of other mechanisms employed by pathogens to counter host immune responses. These mechanisms run the continuum between avoidance and alteration of immune response and include: evasion by entering sites of immune privilege within the host, change or shift in surface expressed antigen or general phenotype, latency, active modification of host immunity via specialized secretion systems, inhibition of uptake and host signaling, and blockade of innate processes along with the down regulation of host immune responses [159]. Further discussion of these general methods, along with specific examples, follows.

Both the parasite *Toxoplasma gondii* and the pathogenic fungus *C. neoformans* are known to have the ability, particularly in immunocompromised individuals, to enter the central nervous system (CNS) [160,161]. This can lead to protracted and serious infection and represents a method of host immune evasion by exploiting immune privilege.

In an article by van der Woude and Baumler, several types of bacterial phase variation are discussed that allow for a circumventing of host immune response [162]. Among these is an ability for *Helicobacter pylori* to vary LPS structure in such a fashion

as to mimic human blood group antigens. While not a naturally occurring phase variation, after serial passage in egg yolk, *C. burnetii* can undergo a gene deletion leading to a phase variant lacking O-antigen (phase II) [163,164,165]. Importantly, it has been suggested that the naturally occurring phase variant (phase I) of *C. burnetii* exhibits a high degree of pathogenicity, due in part to a shielding from host immune activation provided by full-length LPS containing O-antigen [166]. Similarly, induced modification in LPS structure by some species of *Salmonella* has been shown to increase the ability for intracellular survival of the phase variant [167]. *C. neoformans* also undergoes a phenotypic switch in the type of capsule produced, resulting in either a smooth or mucoid colony morphology [168]. Pietrella et. al. [168] showed that these phenotypic differences have immunological impacts, with mucoid colonies being less susceptible to host cell phagocytosis. It has further been shown that encapsulation of *C. neoformans* serves to downregulate host IFN-γ responses [169].

TB is perhaps the prototypical example of immune evasion of bacteria by latency, with the ability to survive for extended periods of time within the infected host in a quiescent non-replicative state [170]. *C. neoformans* is also known to enter latency within the host and the mechanisms involved in the persistence of infection are the focus of ongoing research. However, it is known that latency develops during a state of immunocompetence in the host, and recrudescence of disease is possible in the case of loss of immunocompetence [171]. The AMs, and more generally monocytes, are under investigation as being possible contributors to fungal persistence and facilitators of fungal dissemination [172]. Similarly, the ability for *C. burnetii* to enter latency during chronic

infection has been recognized for decades [173]. The mechanisms involved in this latency are still poorly understood and represent an area of ongoing research.

It has been speculated that one of the additional causes for the observed increased phase I *C. burnetii* pathogenicity, relative to phase II, has to do with the ability to alter the host mechanism of phagocytosis. Specifically, it is thought that phase I *C. burnetii* is able to avoid interaction with the host CR3 and thereby does not fully activate the host cell upon phagocytosis [81,174]. This marks a change in host uptake mediated by a phenotypic variation in the infecting pathogen.

Specialized secretion systems allow for pathogenic modification of the host cell environment. *Pseudomonas aeruginosa* is known to use a type III secretion system to secrete a toxin (ExoS) that is capable of disrupting host cellular signaling resulting in cell death, particularly of host macrophages [170]. *C. burnetii* possesses a type IV secretion system that is used to secrete bacterial-specific proteins into the phagosomal and cytosolic compartments of the host cell facilitating bacterial replication [175]. Additional research indicates that *C. burnetii* uses the type IV secretion system to excrete protein factors involved in blocking host cell apoptosis. *C. burnetii* further modifies cytokine production and phagosomal architecture within the host cell, suppressing host immune responses and providing a more conducive host environment in which to persist [176,177]. The alteration of the phagosome, into what is referred to as a parasitorphorous vacuole, includes a physical expansion of the phagosome and a lowering of the internal pH within the vacuole. Both of these alterations are necessary to allow bacterial replication to occur and both of these alterations are directly facilitated by bacterial

proteins secreted into the host phagosome through the *C. burnetii* type IV secretion system [178,179]. *C. burnetii* has been shown to actively interact with and delay the progression of host cell apoptosis and autophagy [180,181,182]. Thus, the bacteria modify the host cellular environment to allow for both replication and survival. While *C. neoformans* does not possess an analogous secretion system, it has been shown to alter the membrane integrity of the host cell phagosome [183]. It is thought that this breach in phagosomal integrity may facilitate intracellular survival and replication of the fungus and may be mediated in some fashion by the presence of polysaccharide containing vesicles that appear to accompany the disruption of the phagosomal membrane. Thus, while through a different mechanism than that employed by *C. burnetii*, *C. neoformans* also has the capability of altering host immune response in a manner conducive to survival. After alteration of the phagosome and continued replication, *C. neoformans* also possesses the ability to completely escape the host phagosome. This "extrusion" process has also been referred to as vomocytosis and results in the escape of *C. neoformans* from the host phagocyte, while leaving the phagocyte intact [184,185]. Interestingly, it has been shown that antibody-mediated phagocytosis leads to *C. neoformans* biofilm formation upon vomocytosis; whereas, complement-mediated phagocytosis leads to increased systemic fungal dissemination upon vomocytosis [186]. This represents an extreme in pathogen capability to modify and subvert host immune response and further highlights the complex interactions between host and pathogen.

Recent work with *C. neoformans* suggests another fungal strategy for survival includes active induction of the host autophagy pathway [187]. Through this fungal

interaction with the host and subsequent induction of host autophagy, it appears that fungal replication is enhanced. Increased rates of fungal vomocytosis have also been observed in cells induced to undergo autophagy, as compared to vomocytosis rates observed in cells where autophagy progression had been blocked [188]. It appears that *C. neoformans* benefits from host autophagy and may be able to actively induce beneficial autophagy via fungal interactions with the host cell.

Summary of the Introduction

C. burnetii and *C. neoformans* are both able to enter, survive and replicate within the macrophage. Both pathogens are then able to escape from the macrophage and establish systemic infection within the host. Adaptive immunity has been shown to be critical in the ultimate control of both these pathogens. While there is information contained within the literature describing the mechanisms of specific interactions between both *C. burnetii* and *C. neoformans* and macrophages, resulting in both immune evasion and pathogen control, relatively little is known about the interactions between these pathogens and specific macrophage populations *in vivo*. The experiments presented in chapters 2 and 3 were designed to answer questions about what specific macrophage populations are susceptible to infection with either *C. burnetii* or *C. neoformans* and to what extent these macrophages are a permissive replicative niche for infection. In total, the study addresses the role of the AMs during early establishment and maintenance of pulmonary infection and compares this role in the context of either *C. burnetii* or *C. neoformans* infection to the role played by the recruited monocyte and the extracellular milieu.

CHAPTER TWO

RESIDENT ALVEOLAR MACROPHAGES ARE SUSCEPTIBLE TO AND PERMISSIVE OF COXIELLA BURNETII INFECTION

Contributions of Authors and Co-Authors

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Contributions: Conceived and designed the experiments, contributed reagents/materials/analysis tools, critically read and edited the manuscript

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Abstract

Coxiella burnetii is the causative agent of Q fever, a zoonotic disease with potentially life-threatening complications in humans. Inhalation of low doses of *Coxiella* bacteria can result in infection of the host alveolar macrophage (AM). However, it is not known whether a subset of AMs within the heterogeneous population of macrophages in the infected lung is particularly susceptible to infection. We have found that lower doses of both phase I and phase II Nine Mile *C. burnetii* multiply and are less readily cleared from the lungs of mice compared to higher infectious doses. We have additionally identified AM resident within the lung prior to and shortly following infection, opposed to newly recruited monocytes entering the lung during infection, as being most susceptible to infection. These resident cells remain infected up to twelve days after the onset of infection, serving as a permissive niche for the maintenance of bacterial infection. A subset of infected resident AMs undergo a distinguishing phenotypic change during the progression of infection exhibiting an increase in surface integrin CD11b expression and continued expression of the surface integrin CD11c. The low rate of phase I and II Nine Mile *C. burnetii* growth in murine lungs may be a direct result of the limited size of the susceptible resident AM cell population.

Introduction

Coxiella burnetii, an NIAID category B priority pathogen and select agent, is the bacterial pathogen responsible for the clincal symptoms of the zoonotic disease Q fever. In humans, this febrile disease is frequently acquired via inhalation and is characterized

by a flu-like illness which can progresss to pneumonia with severe complications resulting from occurrences of chronic infection. Various strains of *C. burnetii* are recognized. Strain designation was historically determined by either geographical location or clinical isolation of bacterial variants. However, molecular methods have determined that strains are also separated by unique grouping characteristics at the genetic level, such as restriction length polymorphism and plasmid content. Yet it was also noted in these studies that this genetic variation does not directly correlate to virulence; therefore, it was suggested that virulence may be more directly attributable to host response [5,164]. Bacterial phase variants are also recognized irrespective of strain type. Phase I *C. burnetii* is characterized by the presence of O-antigen in the bacterial LPS coat. After serial passage, in egg-yolk or cell culture, gene deletion may occur leading to loss of O-antigen, resulting in the formation of phase II *C. burnetii* [165,189].

In light of the potential role in virulence played by host response to infection, it is critically important to gain an understanding of early immune responses to infection. Despite this need, a clear understanding of the host innate immune response to pulmonary infections with *C. burnetii* has, thus far, been lacking. The macrophage is the cell-type most responsible for harboring *C. burnetii* and facilitating replication within a parasitophorous vacuole [190]. Moreover, macrophages accumulate within the lung during infection, resulting in phenotypic heterogeniety within AMs [73]. However, the AM phenotype marking the population permissive to *Coxiella* growth is not known.

Resident AM are characterized by high levels of CD11c surface integrin expression. This high level of integrin expression is dependent upon the lung

microenvironment, as adoptive transfer of CD11c-/CD11b^{+} peritoneal macrophages into the lung of recipient mice, via intratracheal instillation, results in an upregulation of CD11c [41]. Similarly, monocytes recently recruited to the lung during inflammation initially express the typical monocytic lineage marker CD11b [191]. While AM from the naïve lung do not typically express observable levels of CD11b, certain stressors, such as hyperoxic conditions within the lung, can cause AM activation and subsequent upregulation of CD11b [191,192]. Therefore, during infections, lung macrophage heterogeneniety can result from both an influx of recruited macrophages and changes in cell marker expression by non-elicited cells. Within this heterogeneous mix of resident and recruited cells, there exists a population of CD11c^{-}/CD11b^{+} recruited cells, and a population of CD11c^{+}/CD11b^{-} resident cells, along with the newly observed subset of CD11c^{+}/CD11b^{+} resident cells.

In addition to the high levels of CD11c expression on resident AM, these cells are remarkable for a high degree of autofluorescence when viewed with fluorescent microscopy or analyzed with fluorescence-activated cell sorting (FACS). This combination of CD11c expression and autofluorescence is recognized as characteristic of AM [193,194], and allows for identification of resident AM within the naïve lung. However, upon infection, these characteristics do not provide discriminatory power sufficient to differentiate resident and recruited AM.

Discrimination of resident and recruited macrophages within the heterogeneous lung environment can be accomplished utilizing fluorescent lipophilic dye-labeling [195]. Briefly, the principle of this dye-labeling technique relies on the observation that AM

turn-over rates within the murine lung vary from a half-life of one year under steady-state conditions to a half-life of two months during infection [71]. In contrast, the half-life of circulating murine monocytes has been reported to be twenty-two hours, and the average total time in circulation has been estimated to be forty-eight hours. [57,196]. However, the amount of time monocytes remain in circulation is shortened under inflammatory conditions, with tissue recruited monocytes originating from the circulating population of monocytes [197]. Therefore, a dye-labeling strategy which introduces dye through intra-venous injection, labeling all cells present within the animal and then providing sufficient time for turn-over of circulating cells, will result in a selectively dye-labeled population of tissue resident cells.

In this study, we employed lipophilic dye-labeling to identify the importance of a resident population of macrophages within the lung in initial infection with *C. burnetii*. This population of AM was characterized by surface expression of both CD11c and CD11b and was comprised of long-term resident and newly resident macrophages responding to *C. burnetii* infection. This population was observable in a dose dependent fashion between six and forty-eight hours post-infection (PI) and was responsible for the majority of early cellular uptake of phase II Nine Mile *C. burnetii*. This subset of resident cells was also present in the context of phase I Nine Mile *C. burnetii* infection and the data presented conclusively supported a role for $CD11c^{+}$ resident cells in the initiation and maintenance of phase I Nine Mile infection.

While recruited cells entering the lung subsequent to infection were capable of clearing phagocytosed bacteria, this was not true of resident AM. This cell-type not only

was responsible for initial uptake of the bacteria, it was unable to clear bacteria during the subsequent infection. The result was a replicative niche for bacterial infection which influenced the overall course of infection. This was true irrespective of the phase variant responsible for infection and was not dependent on differential inflammatory responses induced by increasing doses of bacteria.

Results

Low Dose NMII Infection Resulted in Greater Increases in Numbers of Bacteria Within the Lung, Compared to Inocula Numbers, Relative to High Dose Infection

To assess whether resident AM could provide a niche exploited by bacteria during *C. burnetii* infection, quantitative RT-PCR was employed to determine growth rates of either $1x10^3$, $1x10^5$ or $1x10^7$ Nine Mile phase II *C. burnetii* (NMII) in lung tissue of groups of C57BL/6 mice 9 days PI. The results are shown as Log10 change in NMII burden (Log (Endpoint bacterial genome copies per lung/Inoculum)). Thus, the zero baseline represents the inoculum quantity for each respective group. As shown in Figure 2-1, when expressing pulmonary *Coxiella* burdens as the change in genome copies from the inocula number used to infect, mice infected with the lowest dose ($1x10^3$) exhibited the greatest increase in *Coxiella* burdens at each time point. Mice given $1x10^5$ *Coxiella* exhibited less increase in *Coxiella* burden than those given the lowest dose but more than the mice given the highest dose. Mice given the highest dose exhibited an overall decrease in *Coxiella* burdens, relative to the inocula number. (ANOVA $p<0.05$) We obtained similar results in BALB/c mice at 2, 9, 16 or 24 days PI (Figure 2-2).

These data were suggestive of a limited cellular niche present within the lungs that was susceptible to and permissive of infection. At higher infectious doses, bacteria appeared to completely fill this niche and bacteria that did not gain entrance to this niche were forced into cellular populations less conducive to survival and replication. The net result was reduced relative bacterial growth and increased bacterial clearance at higher initial inocula quantities.

It was possible that the results shown in Figure 2-1 could have been the result of high dose infections causing more bacterial dissemination from the lungs relative to low dose infections. However, we found that there were no significant differences in dissemination of NMII *Coxiella* from the lungs to the spleens of BALB/c mice, as indicated by spleen weights, at any of the infective doses used (Figure 2-3). Indeed quantitative RT-PCR analysis of spleens indicated that no phase II *Coxiella* was present in the spleens of any of the mice (results not shown). In addition, there was little effect of dose on disease severity as indicated by similar body weight changes in the three groups of mice given the different doses of NMII (Figure 2-3).

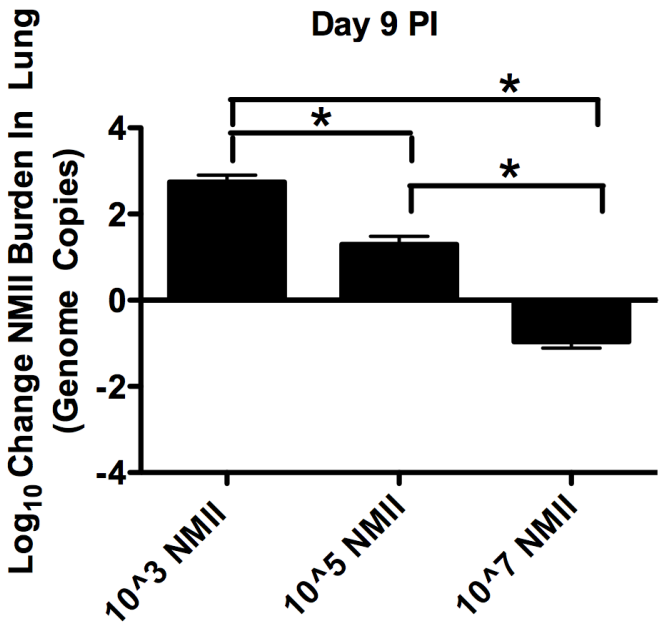


Figure 2-1- Lower doses of NMII resulted in greater relative bacterial numbers in lungs of C57BL/6 mice.

NMII Coxiella bacterial burdens in lung tissue in groups of 4 C57Bl/6 mice infected with $1x10^3$ or 10^5 or 10^7 bacteria in the inocula were assessed, 9 days PI by quantitative RT-PCR. Data is expressed as Log10 change in total genome copies per lung. Significant increases in the final bacterial burdens were observed for both the $1x10^3$ and 10^5 groups, while a significant decrease in the final bacterial burden was observed for the $1x10^7$ group (ANOVA $p<0.05$).

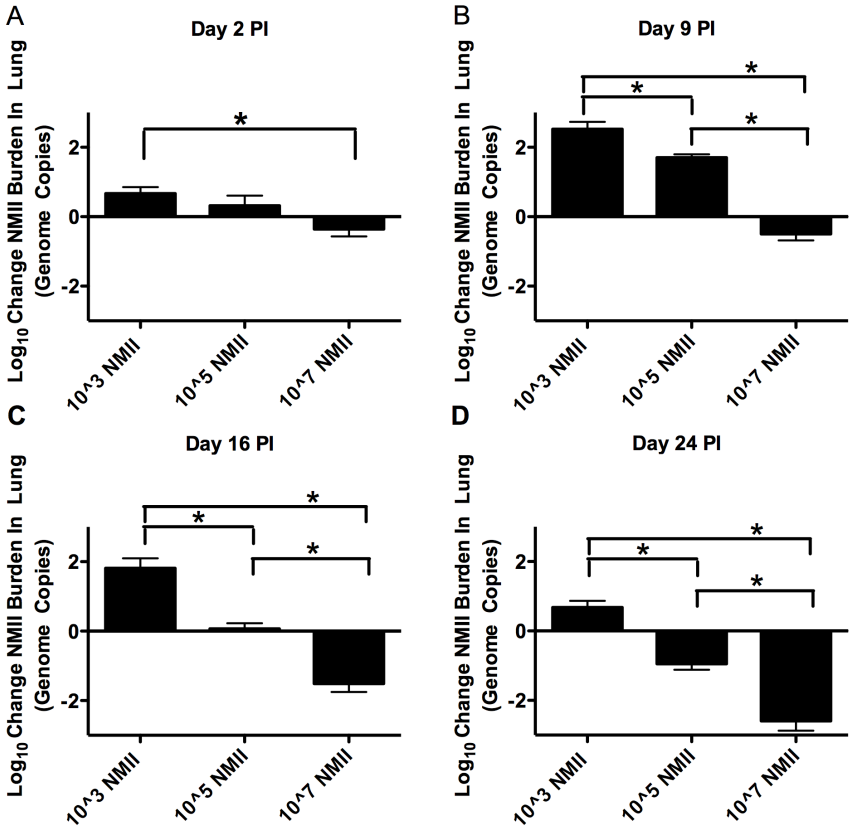


Figure 2-2- Lower doses of NMII resulted in greater relative bacterial numbers in the lungs of BALB/c Mice.

BALB/c mice were infected with either 10^3, 10^5 or 10^7 NMII Coxiella. Bacterial burdens in lung tissue were assessed 2 (A), 9 (B), 16 (C) or 24 (D) days PI by quantitative RT-PCR. Data is expressed as Log10 change in total genome copies per lung (Endpoint bacterial genome copies per lung/Inoculum). Groups were comprised of 4-5 mice with Mean and SEM plotted for each group. Relative to all other inoculum concentrations, the 10^3 group showed a significant increase in bacterial burden across the timecourse of infection. The 10^5 group showed a significant initial increase in bacterial burden and a subsequent significant reduction in overall clearance of bacteria, relative to high dose infections. The 10^7 group showed significant bacterial clearance relative to the other two doses from 9 days PI onward (ANOVA $p<0.05$).

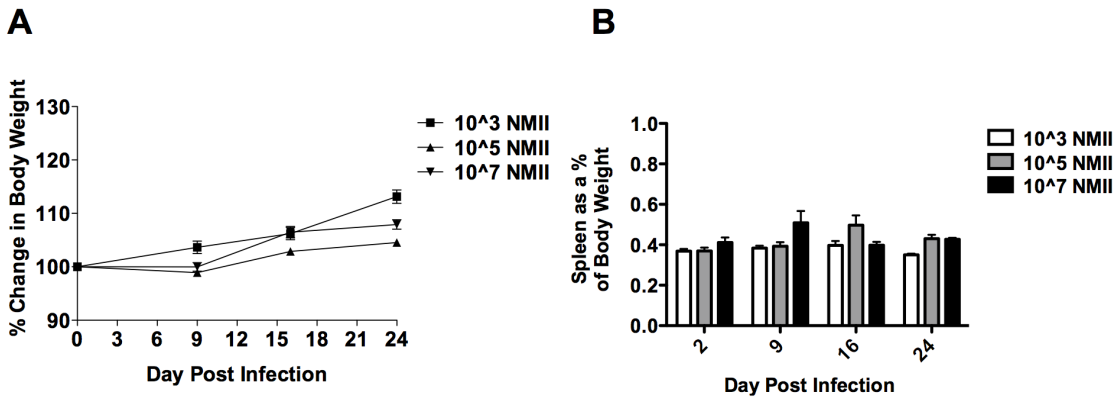


Figure 2-3- High dose NMII infection did not result in increased clinical symptoms of systemic infection in BALB/c Mice.

A) BALB/c mice were infected with either 10^3, 10^5 or 10^7 NMII *Coxiella*. Body weights were recorded daily from day 0 to day 24 PI. Groups were comprised of 4-5 mice with Mean and SEM plotted for each group. Although, there were small significant differences in final body weights among the three doses, none of the doses lost weight across the course of the infection. B) BALB/c mice were infected with either 10^3, 10^5 or 10^7 NMII *Coxiella* and spleen weights were taken at each endpoint (days 2, 9, 16 and 24 PI). Groups were comprised of 4-5 mice with Mean spleen weight as a percentage of body weight and SEM plotted for each group. There are no significant differences between groups or across the timecourse of infection.

NMII Infection Induced Cellular Recruitment
And Phenotypic Change Within AMs in a
Dose and Time Dependent Manner

To understand the kinetics of phenotypic changes of lung macrophages, the surface expression of both CD11c and CD11b integrins on AM (as defined by forward and side-scatter gating) was assessed by FACS on bronchoalveolar lavage fluid (BALF) cells at time-points directly following infection of groups of 4 C57BL/6 mice with low dose ($1x10^3$) or high dose ($1x10^6$) *C. burnetii*. For this experiment, the low infective dose was chosen to match the previous experiment ($1x10^3$ NMII). The high infective dose was chosen to be $1x10^6$ NMII, because within the 48 hr timeframe of the experiment, based on our results from the previous experiment, we felt that this dose would result in zero net change in bacterial burdens relative to the inocula quantity and thus provide the most appropriate comparison dose. Tissue resident AM, prior to infection (0 hrs), exhibited high levels of CD11c expression (Figure 2-4), and these high levels of expression were observed at all time-points and doses subsequent to infection on two AM subsets, the $CD11b^-/CD11c^+$ and $CD11b^+/CD11c^+$ AM. At the low dose (10^3), by forty-eight hours, CD11b expression was upregulated on the $CD11c^+$ population, and $CD11b^+/CD11c^-$ macrophages had accumulated. At the higher dose (10^6), already by twenty-four hours, CD11b expression was upregulated on the $CD11c^+$ population, and $CD11b^+/CD11c^-$ macrophages had accumulated. Thus, there was a dose and time dependent appearance of both a $CD11b^+/CD11c^+$ and a $CD11b^+/CD11c^-$ subset of cells within the overall AM population leading to the observed heterogeneity of the AM population during infection.

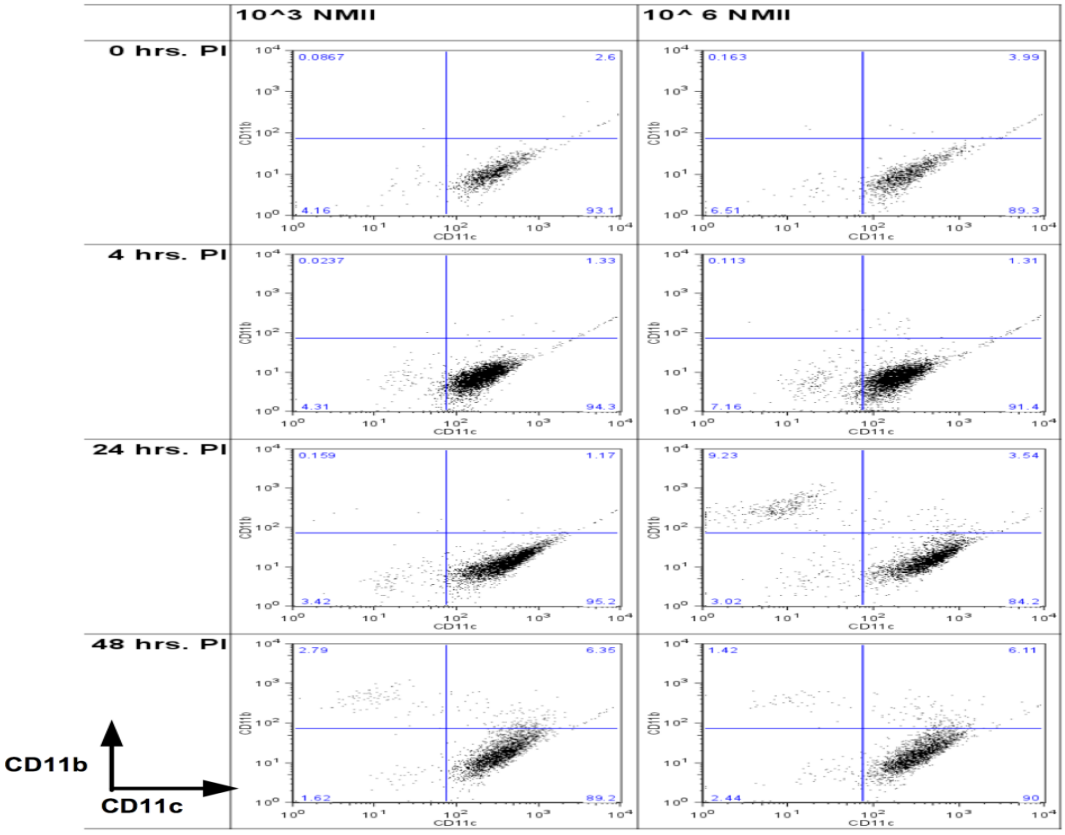


Figure 2-4- Pulmonary infection resulted in time and dose dependent alveolar macrophage phenotypic changes.

Groups of 4 C57BL/6 mice were infected with either 10^3 or 10^6 NMII. Prior to infection, tissue resident AM in these mice were labeled with i.v. injection of the lipophilic dye DiD (see Figure 2-5 for further analysis). Mice were sacrificed 0, 4, 24 or 48hrs PI. Resultant representative dot-plots of forward/side-scatter gated macrophages are shown. Uninfected mice exhibited a typical $CD11b^-/CD11c^+$ resident AM phenotype (top row of figure). Moving L-R in the second row of the figure, representative dot-plots are shown for mice infected 4 hours with 10^3 or 10^6 NMII respectively. Moving L-R in the third row of the figure, representative dot-plots are shown for mice infected 24 hours with 10^3 or 10^6 NMII. A $CD11b^+/CD11c^-$ and a $CD11b^+/CD11c^+$ population of cells were present in the 10^6 infection at this time. Moving L-R in the bottom row of the figure, representative dot-plots are shown for mice infected 48 hours with 10^3 or 10^6 NMII. Both infective doses exhibited a $CD11b^+/CD11c^-$ and a $CD11b^+/CD11c^+$ population at this time.

During NMII Infection, $CD11b^-/CD11c^+$ and $CD11b^+/CD11c^+$ AM Populations were Predominantly Comprised of Cells Resident Prior to Infection, While $CD11b^+/CD11c^-$ macrophages Were Cells Recruited After the Onset of Infection

The time-course of macrophage phenotypic response to pulmonary *C. burnetii* infection, presented in Figure 2-4, suggested that the $CD11b^+/CD11c^+$ macrophage population seen after infection was the resident population, and the $CD11b^+/CD11c^-$ macrophage population was comprised of newly recruited monocytes. To determine the residency or recruitment origins of lung macrophages in the context of infection, mice in the above kinetics experiment were also given intravenous (i.v.) injection of DiD lipophilic dye prior to infection. This lipophilic dye-labeling served to distinguish tissue resident AM from newly recruited macrophages entering the lung during infection. In this way, we were able to both verify AM origin and quantify heterogeneity within the AM subsets phenotypically defined in Figure 2-4.

Macrophages present within the lung during infection were comprised of three main phenotypic subsets, characterized by CD11b and CD11c expression (Figure 2-5). Detectable numbers of $CD11b^+/CD11c^-$ cells had accumulated in the lungs of infected mice by 48 hours, irrespective of dose. None of the $CD11b^+/CD11c^-$ lung macrophages, regardless of time of infection or dose, were DiD positive. Thus, these macrophages were not resident within the lung at the onset of infection. Low numbers of $CD11b^+/CD11c^+$ macrophages were present in the lung before infection; however, this population increased by 48 hours at both infective doses. Regardless of time or dose, approximately half of this cell population was comprised of DiD^+ cells resident within the lung prior to

infection. The combined $CD11b^+/CD11c^+$ and $CD11b^-/CD11c^+$ populations of lung macrophages in low dose infection remained relatively constant in numbers and consisted primarily of DiD^+ cells resident prior to infection. Thus, in the context of low dose infection, the $CD11b^+/CD11c^+$ cell population is predominantly comprised of resident cells that have upregulated CD11b. In contrast, infection with $1x10^6$ NMII resulted in a significant increase in non-resident $CD11b^+/CD11c^+$ cells, relative to resident $CD11b^+/CD11c^+$ cells, at 48 hrs PI. These results are consistent with a larger inflammatory response in the context of higher infective doses.

These results highlighted the upregulation of CD11b in a subset of tissue resident AM responding to infection. Further, these results highlighted a dose dependent recruitment of CD11b cells entering the lung in response to infection. The net result of this recruitment and phenotypic response to infection was a heterogeneous population of $CD11b^+/CD11c^+$ cells present in the alveolus during *C. burnetii* infection.

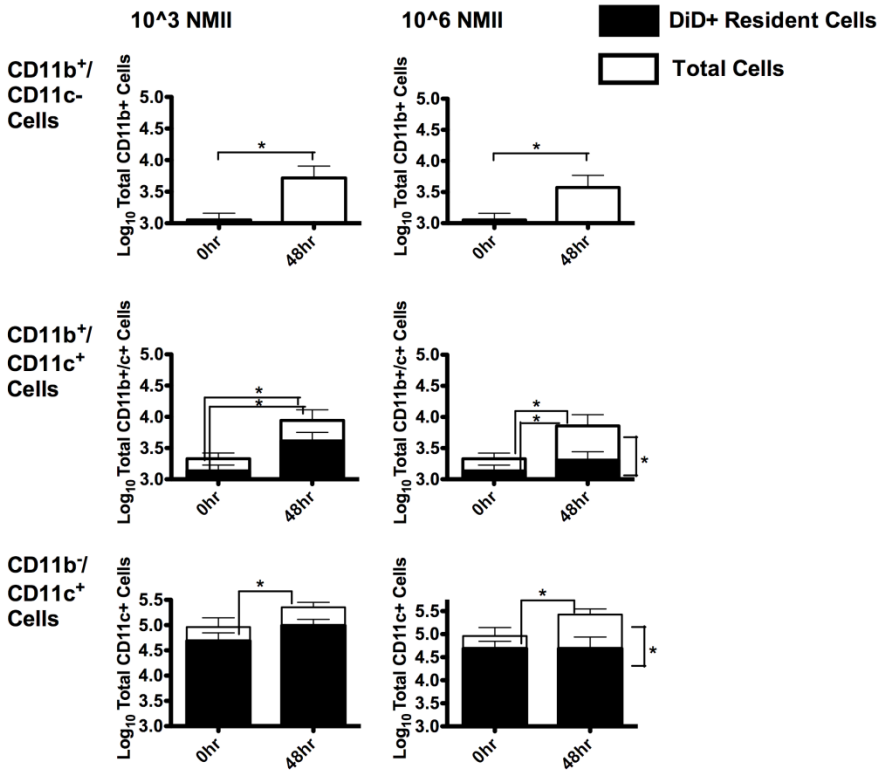


Figure 2-5- Resident AM predominantly expressed CD11c, an AM subset co-expressed CD11b during NMII infection.

The BALF samples collected and presented in Figure 2-4 were further analyzed for the presence of the lipophilic dye DiD. Gated macrophages in the FACS analysis were divided into dye positive and dye negative samples by comparison with an infected unstained control (data not shown). The resultant populations were then expressed as total cell numbers. The results indicated that, AM heterogeneity within the lung, during infection, was characterized by the presence of three main phenotypic AM subsets. Across both the 10^3 and 10^6 infective doses, a significant increase in $CD11b^+$ cells was observed between 0 hrs and 48 hrs PI ($p<0.05$ ANOVA). These cells were newly recruited to the lung, following the onset of infection. There was a heterogeneous population of resident and recruited cells expressing a $CD11b^+/CD11c^+$ phenotype. Finally, there was a resident population of $CD11c^+$ cells that were present within the lung prior to infection. In either infective dose, there was a significant increase in total cells at 48 hrs PI ($p<0.05$ ANOVA).

$CD11c^+$ Resident Cells Were Responsible for NMII Coxiella Uptake and Persistence

To determine whether NMII infection of AMs occurred within the resident or recruited AM population, groups of 5 C57BL/6 mice i.v. injected with $DiIC_{18}(5)$, to label tissue resident cells, were infected with $1x10^7$ GFP NMII (this dose was chosen because, in our hands, GFP expressing *C. burnetii* was slightly less infectious than wild-type *C. burnetii*). At two and nine days PI, BALF cells were subjected to FACS analysis with macrophages identified based on forward and side-scatter characteristics, as well as SiglecF staining [198,199] (Figure 2-6- SiglecF gating not shown). As shown in Figure 2-6A (left-hand column), with respect to the entire AM population, there was a marked increase in the $CD11b^+/CD11c^+$ population from 0 days PI to 9 days PI. This increase was observable as early as 2 days PI, and by 9 days PI the $CD11b^+/CD11c^+$ population predominated in BALF. This increase in the $GFP^+/CD11b^+/CD11c^+$ population was accompanied by a concomitant decrease in the size of the $CD11b^-/CD11c^+$ population across the same time-course.

When the phenotype of only GFP^+ macrophages was assessed, marking those cells having taken up *C. burnetii*, a similar increase in the $CD11b^+/CD11c^+$ population across the time-course of infection was observed (Figure 2-6A- right-hand column). At 2 days PI, both $CD11b^+/CD11c^+$ and $CD11b^-/CD11c^+$ macrophages were positive for the presence of *C. burnetii*. By 9 days PI, GFP^+ macrophages were predominantly $CD11b^+/CD11c^+$.

At 2 and 9 days PI, nearly all of the $GFP^+/CD11b^+/CD11c^+$ and $GFP^+/CD11b^-/CD11c^+$ macrophages were also $DiIC_{18}(5)^+$, indicating that they were tissue resident cells

prior to the onset of infection (Figure 2-6B and Figure 2-6C). However, by 9 days PI, GFP^+ macrophages predominated in the $CD11b^+/CD11c^+$ $DiIC_{18}(5)^+$ resident macrophage population (Figure 2-6B). This is consistent with the above-mentioned phenotypic change (Figure 2-6A) during infection whereby resident AM upregulate CD11b during infection.

These results indicated that the resident AM was both susceptible to and permissive of NMII infection. Further, within the resident AM population, the subset of resident AMs, which underwent a characteristic phenotypic change defined by increased CD11b expression, were the resident AM most highly susceptible to *C. burnetii* infection. Alternatively, the AM population most susceptible to infection upregulated CD11b expression.

Increased Pulmonary Inflammation Was Insufficient to Account for the Observed Deficiency In Bacterial Replication During High Dose Coxiella Infection

To address the possibility that high dose infection was altering the immune status of the resident AM and therefore resulting in the observed reduction in bacterial replication compared to low dose infection, an experiment to block inflammatory cellular influx into the lung during high dose infection was performed. Groups of 5 C57BL/6 mice or CCR2 KO mice having received Gr1-depleting antibody were infected with either 10^3or 10^7 NMII *Coxiella*. The CCR2 KO mice that received Gr1-depleting antibody were anticipated to lack the ability to recruit either inflammatory monocytes or neutrophils to the site of infection.

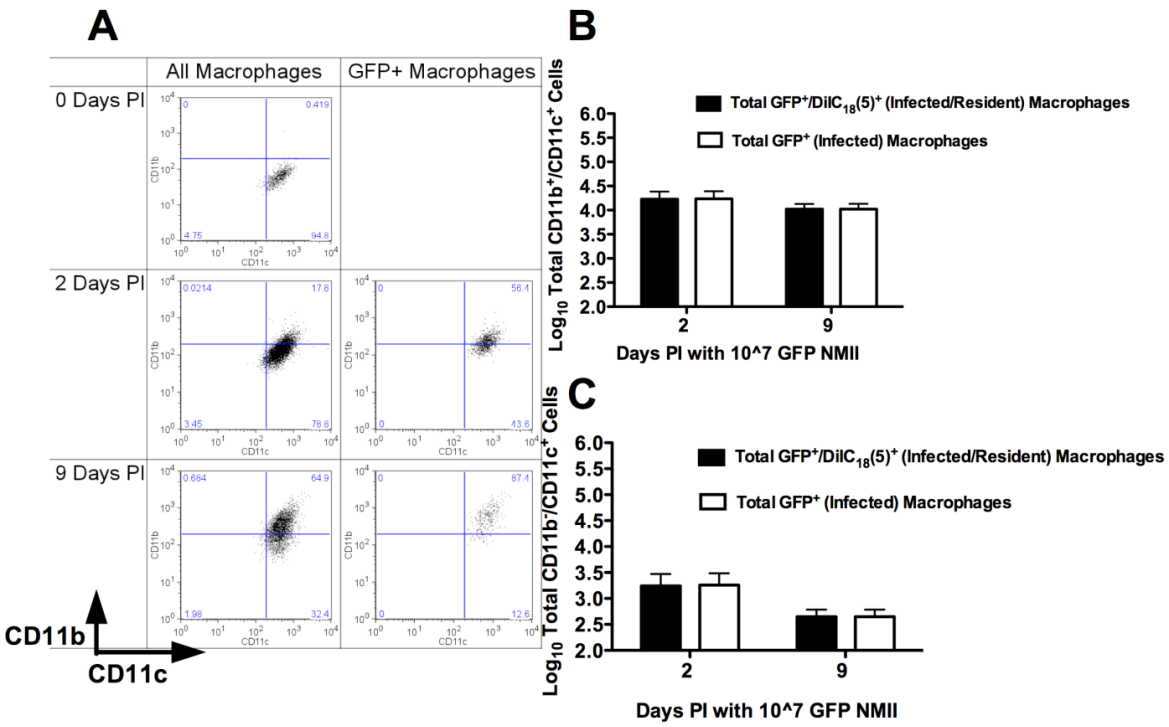


Figure 2-6- CD11b+/CD11c+ AM were the most highly infected cell-type two and nine days post-infection with NMII.

Groups of 5 C57BL/6 mice were infected with $1x10^7$ GFP expressing NMII and sacrificed either 2 or 9 days PI. BALF was collected and FACS was performed on the samples. Representative plots gated for AM on forward/side scatter are shown from groups of 5 mice. A) Beginning at 2 days and peaking by 9 days PI, a significant $CD11b^+/CD11c^+$ AM population was observed in NMII infected animals. It was this population, which predominated as the infected macrophage population. B) The majority of macrophages were $CD11b^+/CD11c^+$ cells. Of these $CD11b^+/CD11c^+$ cells, nearly all were infected cells resident within the lung prior to infection (as determined by $DiIC_{18}(5)$ lipophilic dye staining and GFP fluorescence). There was a minor population of resident $CD11b^-/CD11c^+$ cells that was also infected 2 days PI. This population was not observable 9 days PI.

At day 9 PI, bacterial burdens in lung tissue were assessed by quantitative RT-PCR. As we found previously (Figure 2-1), in the WT mice not depleted of Gr-1$^+$ cells, the low dose NMII resulted in more bacterial proliferation relative to the inocula given than did high dose infection. In the Gr-1$^+$ cell-depleted CCR2 KO mice, the results were similar, with the low dose resulting in relatively more growth of NMII, compared to the high dose. In low dose infection, no significant difference in the change in bacterial burdens was observed between wild-type and Gr-1$^+$ cell-depleted CCR2 KO mice. Both groups exhibited greater than a 2-log increase in bacterial burdens over the inoculum number (Figure 2-7A). This similarity in results, regardless of blockade of inflammatory cell influx, could be due to the minimal overall influx of inflammatory cells during low dose infection (Figure 2-7C and Figure 2-7D). In the high dose infection, there was a significant decrease in both recruited neutrophils (Figure 2-7C) and recruited monocytes (Figure 2-7D) in the Gr-1$^+$ cell-depleted CCR2 KO mice. This blockade of inflammatory cell influx did result in less of an overall reduction in bacterial burdens from the inoculum number in the Gr-1$^+$ cell-depleted CCR2 KO mice, compared to wild-type mice. Importantly, any increase in bacterial replication due to the blockade of inflammatory cell influx was insufficient to increase final bacterial burdens in these animals beyond the initial inoculum number. Thus, differences in inflammation between high dose and low dose infection could not account for all of the differences in growth of *Coxiella* observed between the low and high dose infections. In addition, the results indicate that in the mouse, the influx of new macrophages into the lungs does not supply a source of cells susceptible to NMII infection, further supporting that resident AM are

the susceptible macrophage phenotype (Figure 2-7A). Both low and high dose infection of KO mice receiving depleting antibody treatment resulted in measurable bacterial burdens in spleen tissue 9 days PI; whereas, wild-type mice at either infectious dose did not exhibit bacterial burdens in the spleen at the same time-point (Figure 2-7B). This indicates that inflammation plays a critical role in limiting the dissemination of NMII from the lungs to the spleen. Gr1-depletion did indeed result in a significant reduction in the number of neutrophils recruited to the lung at day 9 PI in high dose infection (Figure 2-7C). Likewise, CCR2 KO animals did exhibit a significant decrease in the number of recruited $Ly6c^{+}/CD11b^{+}/CD11c^{+}$ monocytes at day 9 PI in high dose infection (Figure 2-7D).

Taken together, these data are consistent with the resident AM providing a limited niche for both the establishment and maintenance of NMII infection. These data are not consistent with the idea that alteration of resident AM function resulting from inflammation in high dose infection is sufficient to account for the entire observed reduction in bacterial replication in the context of high dose infection. Further, these data suggest that recruited inflammatory cells and particularly inflammatory monocytes did not act as a pool of cells in the lungs susceptible to NMII infection, but in fact these cells played a minor role in the control of NMII infection. This control could be due either to the ability of inflammatory monocytes to directly clear bacteria present in the lung, or as a result of inflammatory monocytes blocking bacterial dissemination.

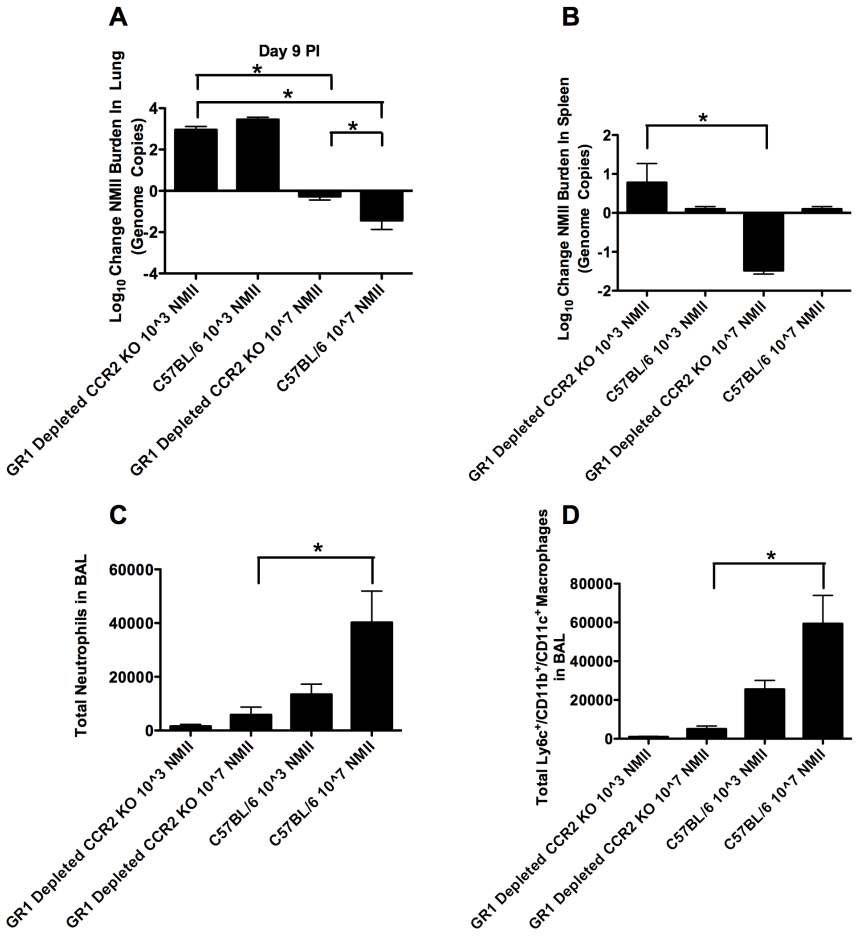


Figure 2-7- Inflammation is not sufficient to account for decreased relative bacterial numbers in higher dose infection.
A) C57BL/6 mice or CCR2 KO mice receiving Gr1-depleting antibody were infected with either 10^3 or 10^7 NMII *Coxiella*. Bacterial burdens in lung tissue were assessed 9 days PI by quantitative RT-PCR. Data is expressed as Log10 change in total genome copies per lung. Groups were comprised of 5 mice with Mean and SEM plotted for each group. Low dose infection of both wild-type mice and KO mice receiving depleting antibody treatment resulted in greater bacterial replication relative to either wild-type mice or KO mice receiving depleting antibody treatment and high dose infection. B) Either low or high dose infection of KO mice receiving depleting antibody treatment exhibited measurable bacterial burdens in spleen tissue 9 days PI; whereas, wild-type mice at either infectious dose did not exhibit bacterial burdens in the spleen at the same time-point. C) Gr1-depletion resulted in a significant reduction in the number of neutrophils recruited to the lung at day 9 PI in high dose infection. D) CCR2 KO animals exhibited a significant decrease in the number of recruited $Ly6c^+/CD11b^+/CD11c^+$ monocytes at day 9 PI in high dose infection. ($p<0.05$ ANOVA)

Low Dose NMI Infection Resulted in Greater Increases, Compared to Inocula Numbers, in Bacteria Numbers Within the Lung Relative to High Dose Infection

To assess whether resident AM could provide a niche exploited by bacteria during Nine Mile phase I *C. burnetii* (NMI) infection, similar to what we observed for Nine Mile phase II *C. burnetii,* quantitative RT-PCR was employed to determine growth rates of either $1x10^3$ or $1x10^5$ NMI in lung tissue of groups of C57/BL6 mice at 9 days PI. As shown in Figure 2-8, when expressing pulmonary *Coxiella* burdens as the change in genome copies from the inocula number used to infect, mice infected with the lowest dose ($1x10^3$) exhibited a greater increase in *Coxiella* burdens relative to mice given the higher dose ($1x10^5$) (ANOVA $p<0.05$). We obtained similar results in BALB/c mice at 2, 9, 16, or 23 days PI (Figure 2-9).

Interestingly, with NMI infection we were able to isolate bacterial DNA from the spleens of infected BALB/c mice. At day 9 PI, there was a significant increase in splenic bacterial burden for the $1x10^3$ NMI group, relative to the other groups. The changes in bacterial burdens within the spleen, for all groups, appeared to follow a general trend of lagging changes in bacterial burden observed in the lung. This is quite consistent with the idea of pulmonary replication within a limited cellular niche followed by breakout from macrophages and dissemination of the bacteria. In particular, bacterial burdens within the lung exhibited an increase from day 9 to day 16 PI for the mid dose infection (Figure 2-9). At day 23 PI, bacterial burdens in the spleen exhibit an increase, relative to day 16 PI, for the mid dose infection (Figure 2-10). Again, as with NMII infection, these data indicated that high infectious doses exhibited decreased replicative efficiency relative to

low infectious doses. This difference could not be solely accounted for by an increased dissemination from the lung of NMI organisms after high dose infections. However, there does appear to be increased bacterial dissemination at all infective doses, and notably at higher doses, in NMI infection compared to NMII infection. In fact, this may be a key factor contributing to the observed differences in virulence for the two bacterial phases.

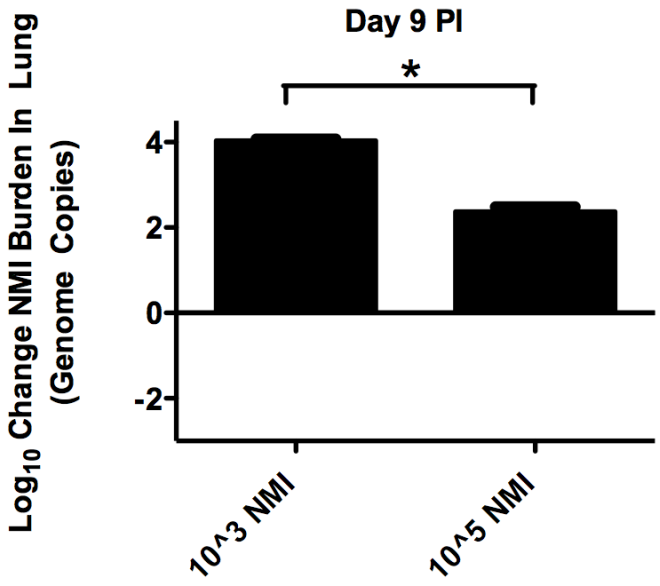


Figure 2-8- Lower doses of NMI resulted in greater relative bacterial numbers in lungs of C57BL/6 mice.

NMI *Coxiella* bacterial burdens were assessed 9 days PI in groups of 5 C57BL/6 mice infected with either $1x10^3$ or 10^5 starting inocula. Bacterial burdens in lung tissue were assessed by quantitative RT-PCR. Data is expressed as Log10 change in total genome copies per lung. A significant increase in the final bacterial burden was observed in the lower starting inocula group for this mouse strain (Student T test $p<0.0001$).

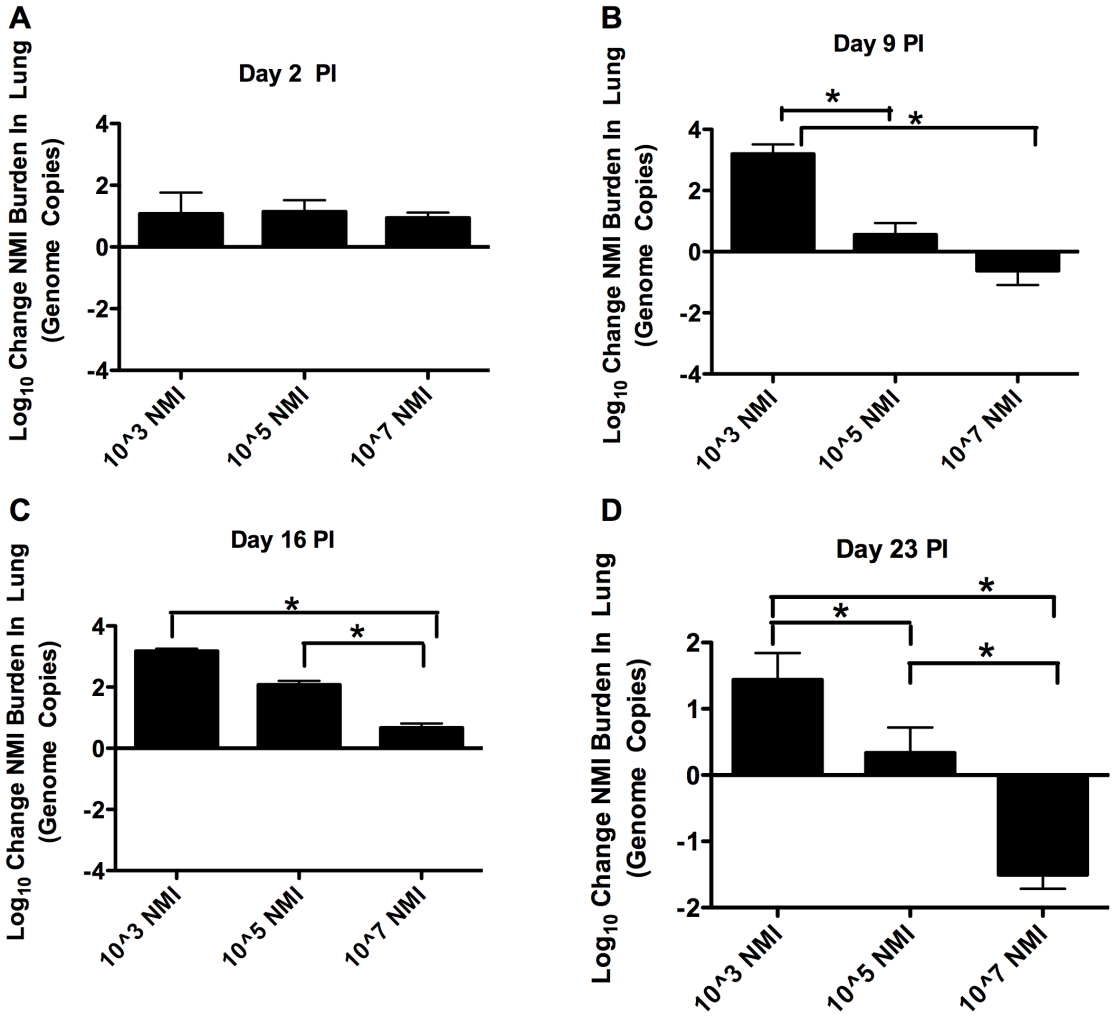


Figure 2-9- Lower doses of NMI resulted in greater relative bacterial numbers in the lungs of BALB/c Mice.

A) BALB/c mice were infected with either 10^3, 10^5 or 10^7 NMI *Coxiella*. Bacterial burdens in lung tissue were assessed 2 (A), 9 (B), 16 (C) and 23 (D) days PI by quantitative RT-PCR. Data is expressed as Log10 change in total genome copies per lung. Groups were comprised of 4-5 mice with Mean and SEM plotted for each group. Relative to the other inoculum concentrations, the 10^3 group showed a significant increase in bacterial burden from 9 days PI onward. The 10^5 group showed a significant increase in bacterial burden, relative to high dose infections from 9 days PI onward. The 10^7 group showed significant clearance relative to the other two doses from 16 days PI onward (ANOVA $p<0.05$).

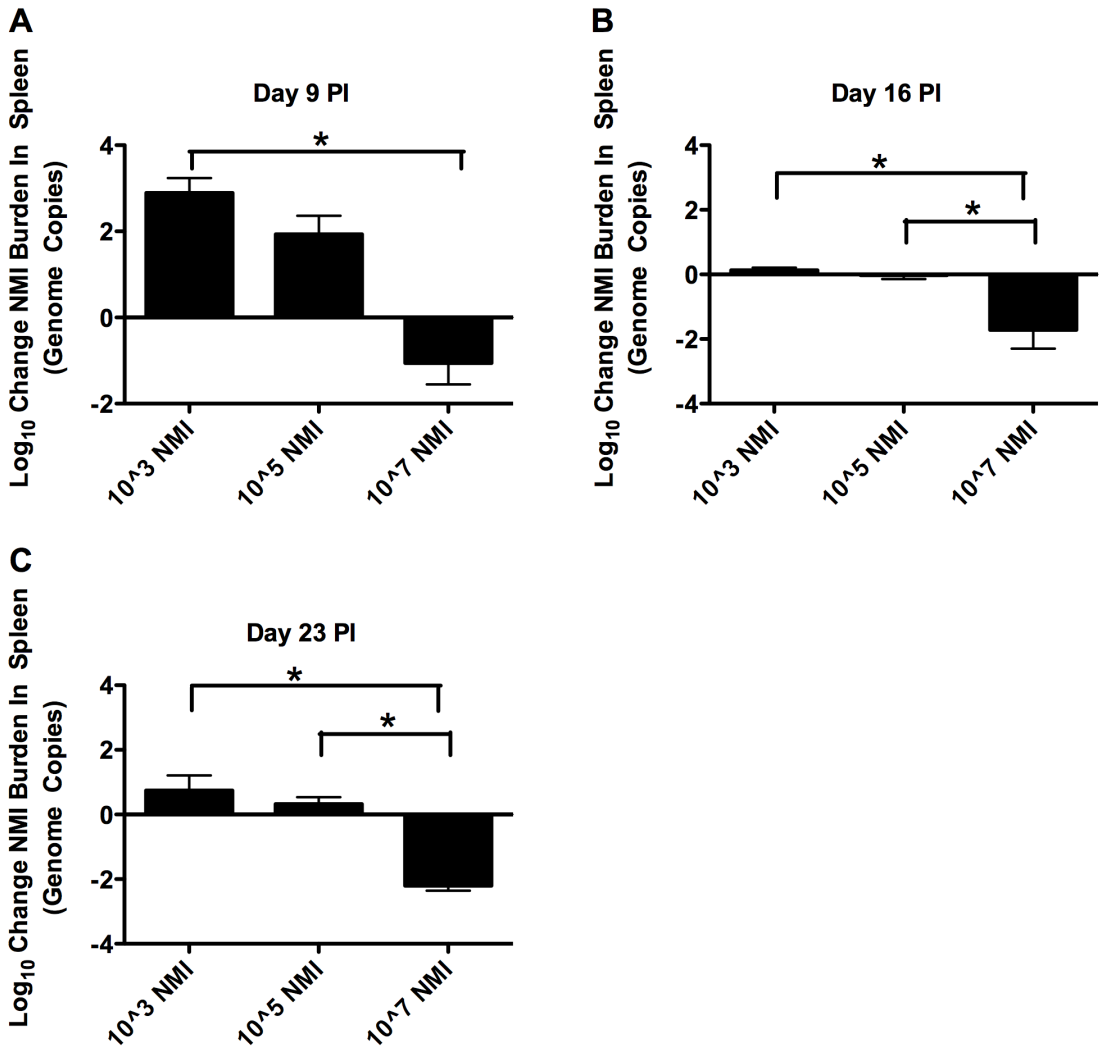


Figure 2-10- Lower doses of NMI resulted in greater relative bacterial numbers in the spleen of BALB/c Mice.

A) BALB/c mice were infected with either 10^3, 10^5 or 10^7 NMI *Coxiella*. Bacterial burdens in spleen tissue were assessed 2 (A), 9 (B), 16 (C) and 23 (D) days PI by quantitative RT-PCR. Data is expressed as Log10 change in total genome copies per spleen. Groups were comprised of 4-5 mice with Mean and SEM plotted for each group. Relative to the other inoculum concentrations, the 10^3 group showed a significant increase in bacterial burden at 9 days PI. The 10^7 group showed significant clearance relative to the other two doses from 9 days PI onward (ANOVA $p<0.05$).

Phenotypic Responses of AM to NMI Infection
Were Similar to Those of AM to NMII Infection,
With $CD11b^+/CD11c^+$ and $CD11b^-/CD11c^+$ AM
Populations Predominantly Comprised of Resident Cells

Having determined both the overall phenotypic profile during infection and the phenotypic profile associated with susceptibility to NMII infection, we determined whether the observed AM phenotypic response was consistent across *C. burnetii* phase variants.

Due to the limited capacity for FACS analysis in our BSL-3 facility, and because a GFP expressing phase I *C. burnetii* was not available, we were not able to directly determine the phenotype of AM infected with NMI by FACS. Thus, we compared the AM phenotypic response in NMII infected mice to that in NMI infected mice. To determine whether the phenotypic responses of AM to *C. burnetii* infection were consistent across phase variants, groups of 5 C57BL/6 mice were infected with low dose ($1x10^4$ – chosen to be intermediate between the two doses causing bacterial replication in the previous experiment and still sufficient to cause an inflammatory response observable by FACS) NMI. Phenotypic changes in CD11b expression during NMI infection were analogous to those observed during the course of NMII infection (Compare Figure 2-6A and 2-11A).

As shown in Figure 2-11A, two days PI, there was a marked increase in the $CD11b^+/CD11c^+$ population of cells, defined as macrophages based on SiglecF expression (parental gating strategy not shown), relative to the naïve control. By nine days PI, this double positive cell-type predominated in the BALF. The progression of phenotypic change from 2 to 9 days PI mirrors the increase in bacterial replication shown

across the same timecourse (Figure 2-9). This further supports the claim that it is specifically the $CD11b^+/CD11c^+$ AM population that is both most susceptible to and permissive of infection.

In a separate experiment, groups of 5 DiD lipophilic dye-labeled C57BL/6 mice were infected with $1x10^4$ NMI and killed at 6 days PI. The CD11b and CD11c staining of the AM (Figure 2-11B) was similar to the staining observed on AM from mice killed at day 9 PI (Figure 2-11A). Further, this $CD11b^+/CD11c^+$ AM population is comprised almost exclusively of tissue resident AM (as assessed by DiD staining and presented in Figure 2-11C).

These results indicated that, with respect to AM phenotypic responses, the two *C. burnetii* phase variants elicited similar immune responses. This was consistent with the previously mentioned speculation that both bacterial phase variants present similarly to the host immune system, and that there is an intrinsic difference in down-stream host immune response that is responsible for the observed differences in virulence. More importantly, this also suggested that, analogous to our findings in NMII infection, the resident AM might provide a required niche for both NMII and NMI infection.

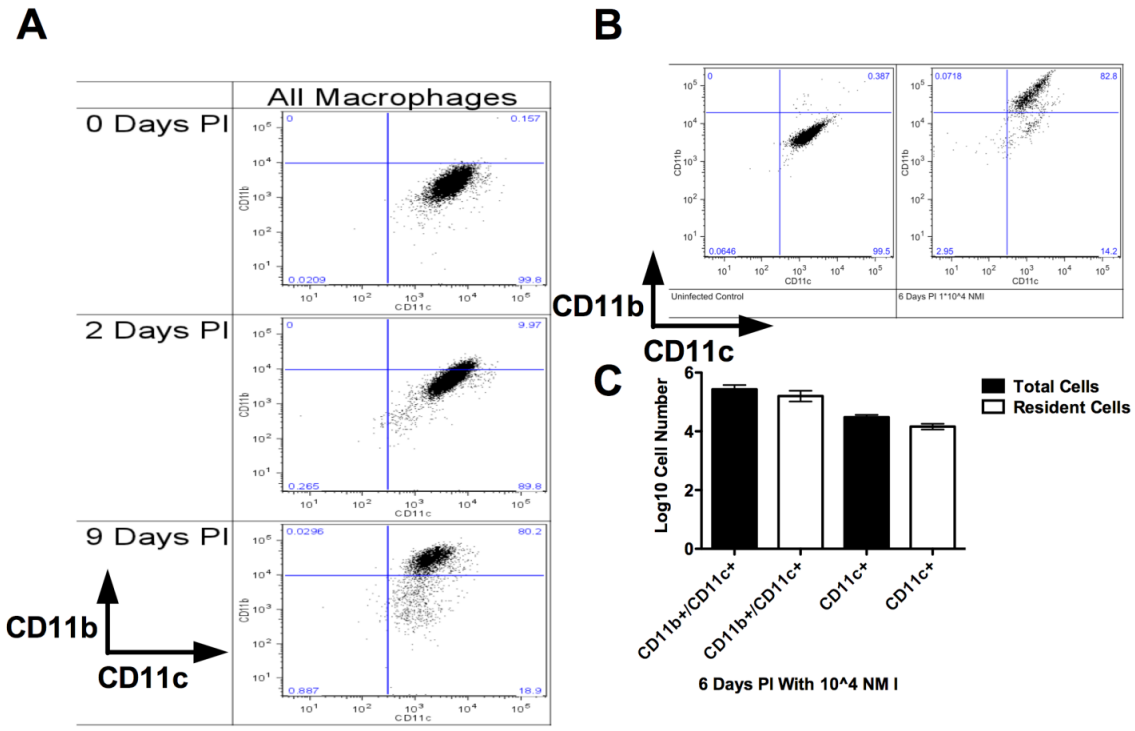


Figure 2-11- Resident AM predominantly expressed CD11c, an AM subset co-expressed CD11b during NMI infection.

A) Two days PI there were $CD11b^{-}/CD11c^{+}$ and $CD11b^{+}/CD11c^{+}$ AM subsets present within the groups of 5 NMI infected C57BL/6 mice. By 9 days PI, the $CD11b^{+}/CD11c^{+}$ AM subset predominated as the major AM subset present within the NMI infected animals. B) By 6 days PI, the $CD11b^{+}/CD11c^{+}$ AM subset was already predominating as the major AM subset present within the NMI infected animals. C) In NMI infection, both the $CD11b^{+}/CD11c^{+}$ and $CD11b^{-}/CD11c^{+}$ macrophage populations were predominantly comprised of cells resident within the lung prior to infection (as determined by DiD lipophilic dye staining). This was analogous to the phenotypic profile observed in NMII infection.

NMI Coxiella Infected Cells Were Phenotypically Consistent With Resident AM

Given the similarities in the characteristic changes in phenotypic expression of AM subsets throughout the course of NMII and NMI infection, we hypothesized that during NMI infection the resident AM would be the cellular subset susceptible to infection. To test this, we infected groups of 5-6 C57BL/6 mice with $1x10^4$ NMI (dose chosen to match the previous experiment) for 12 days. At 12 days PI, cytospin slides of BALF cells from the two groups of mice were prepared and AM were stained for both *C. burnetii* and CD11c expression (Figure 2-12A-representative sample, Figure 8B-negative control).

NMI infected AM at day twelve PI were characterized by CD11c staining. That the infected AM expressed high levels of CD11c, was suggestive of lung residency prior to infection.

Discussion

Our results indicated that a subset of tissue resident AM was both susceptible to and permissive of *C. burnetii* infection. This population of cells underwent a characteristic phenotypic upregulation of CD11b expression subsequent to infection. More importantly, this population represented a limited cellular niche pivotal in the initiation and maintainence of infection. The limited nature of this population may also account for the general limited susceptibility of mice to *C. burnetii* infection.

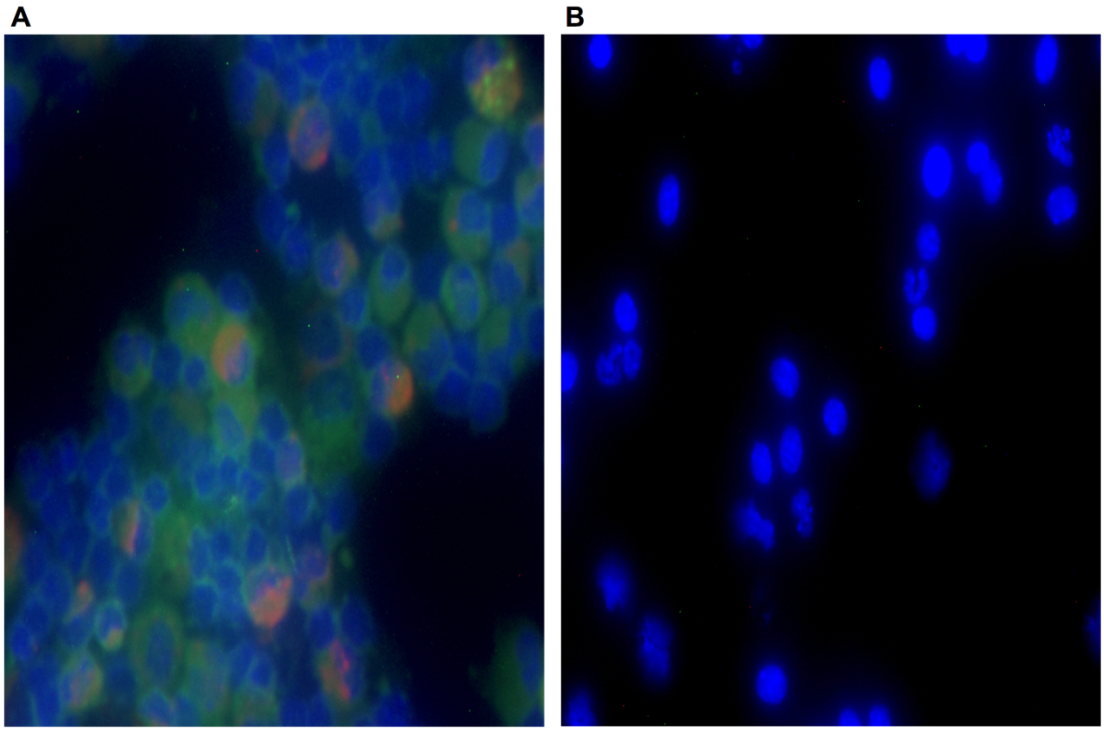


Figure 2-12- NMI infected cells expressed $CD11c^{+}$ and were consistent with a resident AM phenotype.

Groups of 5-6 C57BL/6 mice were infected with $1x10^4$ NMI. Twelve days PI, BALF fluid was obtained and histology of cytospin preparations was performed. A) Twelve days PI with $1x10^4$ NMI, infected cells were identified by the presence of *Coxiella* (stained red) and CD11c expression (stained green). Results were consistent with infected cells being a resident AM phenotype. B) Twelve days PI with $1x10^4$ NMI, BALF cells were adhered to slides and were not stained as a control for autofluorescence. Representative photomicrographs are shown.

C. burnetii has long been recognized as highly infectious, with literature reports suggesting that as few as one organism may be capable of causing infection in humans [4,7]. Moreover, a recent study has shown a strong statistical correlation between bacterial infectivity and the ability to survive intracellularly in immune cells. This study suggested that bacteria with high ID50's overwhelm the immune system through rapid growth, while bacteria with low ID50's evade the immune response [139]. Such an evasion strategy would be very consistent with the characteristics of *C. burnetii* infection and would be consistent with the idea of a limited cellular niche susceptible to and permissive of infection.

Guinea pigs are often used as a model system to study *C. burnetii* infection, as these animals manifest dose dependent symptoms which closely recapitualte disease in humans [200]. However, SCID mice also exhibit enhanced susceptibility to both phase I and phase II *C. burnetii* infection and have been used as a susceptible mouse model of disease [201]. Within animal models of disease, bacterial strain differences affecting susceptibility to infection have been noted [202]. Specifically, while SCID mice appeared susceptible to all *C. burnetii* isolates tested in the study, various groups of isolates exhibited differing levels of infectivity in guinea pigs. In particular, Nine Mile isolates (the isolate used for all studies presented here-in) proved to be infectious regardless of the animal model employed. Moreover, while no wild-type mouse strain is particularily susceptible to phase II *C. burnetii* infection, mouse strain differences have been noted to play a role in overall susceptibility. For example, C57BL/6 mice were shown to be less susceptibile to NMII infection than BALB/c mice [203]. In light of our results in this

study, these differences in susceptibility could be consistent with a difference in the ability to control bacterial break-out from the susceptible AM niche. Further, this would be consistent with previous work performed in our lab showing the requirement for adaptive immunity in the control of *C. burnetii* infection [91]. Thus, while the permissive and susceptible AM population plays a central role in influencing the overall course of infection, the ultimate outcome of infection is determined by the adaptive immune response. The interaction between the early susceptible innate AM population and the later adaptive response is an area for on-going research.

We observed that low doses of NMII were not only cleared less efficiently in early infection, but also appeared to replicate more efficiently, relative to high dose infections (Figure 2-1 and Figure 2-2). This suggested that a cellular niche within the lung was acting to harbor *C. burnetii*, providing an environment conducive to bacterial replication. At low doses most of the bacteria were able to exploit this niche; at high doses some of the bacteria were probably forced into less favorable niches. In deriving this conclusion, it is important to note that we were unable to recover bacteria from the spleens of animals infected with any dose of NMII across any of the timecourse of infection (see Figure 2-3 for surrogate measures of systemic infection). This was a strong indication that our observations of dose dependent differences in bacterial clearance and replication within the lung could not be merely attributed to a greater rate of bacterial dissemination in high dose infected animals. When considering the data from the onset to conclusion of infection, we did not observe any dose-related systemic differences in infection. Instead, we observed a lung localized difference in bacterial burden where low

dose innocula led to increased bacterial burdens relative to the infective dose used. Similar results were obtained in the context of NMI infection, with the notable exception of increased bacterial dissemination across infective doses (data not shown). This is consistent with the increased virulence of the NMI phase variant.

The role which the micro-environment of the lung plays in immuno-regulation of the AM populations, and by extension, the effect it has on the susceptibility of these populations, is becoming increasingly recognized. For example, immunomodulatory interactions of CD200 expressed on airway epithelium, and CD200R expressed on alveolar myeloid cells including AM, downregulates alveolar myeloid cell and AM function [204,205], whereas inflammatory monocytes accumulating in the lungs would not come under this control. Our data suggested that *C. burnetii* may take advantage of the suppressed state of resident AM, preferentially infecting this cell population.

As mentioned, we had reason to believe that *C. burnetii* was exploiting a cellular niche in order to survive, and this niche was likely to be found within the AM population. In this regard, we observed a phenotypic switch in the infected macrophage population such that infected $CD11c^{+}$ cells began to upregulate CD11b surface integrin expression (Figure 2-4). This observation was made during FACS analysis of cells from lavage fluid. This analysis provided a distinguishing phenotypic description of infected cells. However, this analysis alone provided limited data regarding whether the infected cells were tissue resident within the lung prior to the onset of infection. Therefore, this approach was insufficient to identify the putative cellular niche susceptible to bacterial infection within the heterogeneous cellular environment of the lung. Still, the approach

was sufficient to provide direction to future analysis, specifically suggesting that the resident AM could account for a susceptible cellular niche, consistent with our hypothesis.

Macrophage heterogeneity within the alveolus has been recognized for some time. This heterogeneity includes a mixture of $CD11b^{-}/CD11c^{+}$ expressing resident cells and $CD11b^{+}/CD11c^{-}$ expressing recruited cells. Additionally, although literature directly addressing phenotypic changes of lung macrophages during infection is limited [206], we show here that a population of $CD11b^{+}/CD11c^{+}$ cells comprised of both previously $CD11b^{-}/CD11c^{+}$ long-term resident and comparatively newly resident previously $CD11b^{+}/CD11c^{-}$ cells exists within the alveolus of the lung directly following *C. burnetii* infection (Figure 2-5).

To determine whether infected AM were resident in the lungs at the time of infection, resident AM subsets were identified, utilizing a dye-labeling technique. Davidson et. al. found that when introducing the fluorescent lipophilic dye DiI systemically through tail-vein injection, tissue resident macrophages were dye bright in recipient mice six days after injection, and circulating monocytes were dye negative to dye low [195]. Utilizing this method, we were able to discern both resident and recruited macrophage populations within the lung. Then, by employing a GFP expressing NMII bacteria, we were additionally able to identify members of these various phenotypic subsets as either *C. burnetii* infected or non-infected (Figure 2-6). Our findings show that the $CD11b^{+}/CD11c^{+}$ resident AM population observed during infection was the most highly susceptible population. While it is possible that $CD11c^{+}$ cells harboring fewer

bacteria were indistinguishable from the autofluorescent background, the highly infected $CD11b^+/CD11c^+$ population of cells was clearly identifiable.

These findings are consistent with the work of Garn et. al., who found that after NO_2 exposure $CD11b^+$ cells predominate in the lung macrophage population [207]. Here, we not only show an influx of $CD11b^+/CD11c^-$ cells upon induction of infection, we show that these recruited cells upregulate CD11c upon entry into the lung during the transition to resident AM, thus providing an *in vivo* finding within the context of pulmonary infection to corroborate the adoptive transfer work performed by Guth et. al., that concluded $CD11c^-$ monocytes upregulate CD11c upon migration into the lung alveoli [41]. Moreover, and most importantly, we show an upregulation of CD11b by a subset of resident AM subsequent to infection. It is this subset of resident cells which predominates in the early uptake of NMII *C. burnetii* and becomes functionally relevant in the establishment of infection.

Because high dose infection most certainly will result in increased pulmonary inflammation over that induced by low dose infection, we devised experiments to limit inflammation in the context of high dose infection. In this way we could investigate whether the deficiencies in bacterial replication observed during high dose infection were the result of inflammation limiting bacterial growth, as opposed to a saturation of the permissive niche by high dose infection. In our model, we took advantage of CCR2 knock-out mice lacking a functional receptor for monocyte chemoattractant protein-1 (MCP-1). These mice have been shown to be difficent in both monocyte recruitment [208] and clearance of *Listeria monocytogenes*, an intracellular bacterium [209]. While

some diminishment of recruited inflammatory monocytes has been observed with Gr1-depletion [74], we expected this to be minimal. Therefore, Gr1-depletion alone was insufficient to inhibit monocyte recruitment into the lungs upon high dose infection. However, by employing Gr1-depletion in CCR2 KO animals we were able to significantly reduce both neutrophil recruitment and $Ly6c^+/CD11b^+/CD11c^+$ monocyte recruitment (Figure 2-7) while leaving the resident AM population unaltered. The $Ly6c^+/CD11b^+/CD11c^+$ cells have been variously described as inflammatory monocytes, inflammatory dendritic cell precursors, or inflammatory dendritic cells [210,211,212]. Blockade of inflammatory cell influx to the lung did result in significantly less clearance (or enhanced growth) of high dose NMII. However, at the low dose, the absence of inflammation had no significant effect. Overall, the absence of inflammation did not result in a relative increase in growth in the high dose sufficient to effect NMII burdens to the extent observed in the mice receiving low dose infection. Thus, differences in inflammation between high dose and low dose mice could not account for all of the differences in *Coxiella* growth observed between the low and high dose infections. Further, the influx of new macrophages into the lungs did not appear to supply a source of cells susceptible to NMII infection. This observation is consistent with resident AM providing a limited susceptible and permissive niche during infection. This niche appeared to be unchanged in a pro-inflammatory environment. Moreover, these results indicated that the recruited monocyte population played an important role in the control of infection through facilitating bacterial clearance and possibly altering bacterial dissemination from the lung to the spleen.

We recapitulated our NMII findings in the context of NMI infection and showed that low dose infection leads to enhanced bacterial inocula replication within the lung (Figure 2-8). Unlike NMII infection in wild-type animals, but similar to NMII infection in CCR2 KO Gr1-depleted animals, NMI infection resulted in bacterial dissemination to the spleen at all infective doses (Figure 2-10). In fact, this difference in bacterial dissemination could account for the observed differences in virulence between NMII and NMI.

Due to the technical constraints of working with NMI in BSL-3 conditions, we could not directly show that it it is the $CD11b^{+}/CD11c^{+}$ resident AM which is the key cellular niche for phase I infection. However, we were able to conclusively show that it is a $CD11c^{+}$ resident AM which harbors NMI bacteria (Figure 2-12). The phenotypic profiling during the time-course of NMI infection is consistent with that of NMII infection, further implicating the $CD11b^{+}/CD11c^{+}$ population of resident cells as the permissive niche (Figure 2-11).

Both phase II and phase I bacteria exhibited enhanced growth and persistence when infective doses were lower, as seen in the dose response experiments. Importantly, mice did not succomb to infection of either phase at any of the doses used. We observed that the phenotypic response of the AM were similar in both NMII and NMI infection. Moreover, most of the infected AM in NMII infection were cells resident within the lung prior to infection. Likewise, most of the NMI infected cells expresed CD11c and were phenotypically consistent with being resident AM. Furthermore, the dose dependent growth of Coxiella could not be attributed solely to differences in either inflammation or

dissemination. Taken together, this data is highly suggestive that the cellular niche susceptible to and permissive of both phase II and phase I infection is a subset of the resident AM population.

In conclusion, the resident AM provides a pivotal niche involved in maintaining *C. burnetii* infection. Within this resident AM population a subset of cells, characterized by surface expression of both CD11c and CD11b integrins, was primarily responsible for the initial uptake of *C. burnetii*. The role of the resident AM as a susceptible and permissive niche for *C. burnetii* infection was conserved across bacterial phase I and phase II variants. Although *C. burnetii* infection causes an influx of inflammatory macrophages into the lungs of mice, this population was not permissive to *C. burnetii* growth.

Materials And Methods

Ethics Statement

All experimental procedures performed were in accordance with Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee at Montana State University, Bozeman, MT (Protocol permit # 2011-12). All efforts were taken to minimize pain and suffering.

Bacterial Strains

Coxiella burnetii Nine Mile phase I (NMI strain RSA493), Nine Mile phase II (NMII strain RSA439) and Nine Mile phase II GFP (NMII GFP phase II, clone 4 strain

RSA439/Tn7-CAT-GFP) were kindly donated by Robert Heinzen (Rocky Mountain Labs NIH/NIAID, Hamilton, MT).

Animals

Mice used were male mice between 6 and 8 weeks of age and obtained from NCI, Rockville MD or in-house breeding colonies established at Montana State University with founder mice obtained from Jackson Laboratory, Bar Harbor ME. Dose response experiments (Figures 2-2,3,9,10) were performed in BALB/c mice; all other experiments were performed in C57BL/6 mice. Experiments with CCR2 KO mice were performed using mice from an in-house breeding colony established at Montana State University from commercially available founder B6.129S4-Ccr2tm1Ifc/J mice (stock#004999) obtained from Jackson Laboratory, Bar Harbor ME.

Intra-tracheal Infection of Mice

For all experiments, mice were lightly anaesthetized with isoflurane and inoculated intratracheally (i.t.) with final concentrations of NMI or NMII genome copies suspended in 100µl sterile phosphate-buffered saline (PBS).

Collection of Bronchoalveolar Lavage

BALF was collected as previously described [91]. Briefly, mice were given an intraperitoneal (i.p.) injection of a Phenobarbital solution and exsanguinated. Following this, mice were cannulated via a small tracheal incision and the lungs were flushed with aliquots of Hanks' balanced salt solution with 3 mM/liter EDTA in a final volume of 5ml.

Determination of Bacterial
Burden by Quantitative PCR

To assess and compare bacterial burdens in lung tissue under the various experimental conditions, quantitative PCR was performed as previously reported [8,91]. Briefly, whole sample DNA was extracted from lung or spleen tissue with a Qiagen DNeasy blood and tissue kit (Qiagen Inc USA, Valencia, CA) following manufacturer's protocols. Bacterial DNA was amplified in a quantitative real-time PCR reaction with SYBR green PCR master mixture (Applied Biosystems, Foster City, CA). Genome copies were determined by amplification of *rpoS* gene copies as previously reported [8,91]. Reactions were performed with an Applied Biosystems 7500 real-time PCR system (Applied Biosystems).

Calculation of Body Weight and
Spleen as a Percent of Body Weight

Mice were weighed daily on a Denver Instrument Company WE Series 400 digital scale (Denver Instrument Bohemia, NY). Body weights were recorded in grams and % change in body weight was calculated by the following formula: (Final Body Weight (g) / Initial Body Weight (g)) * 100. At each endpoint, spleens were removed and weighed. The resultant weight was recorded in grams and divided by total body weight. This final number was multiplied by 100 and reported as Spleen as a % of body weight.

$Gr1^+$ Cell Depletion

$Gr1^+$ cell depletion was performed by daily i.p. injection with 250μg RB6-8C5 hybridoma derived GR1-depleting antibody produced in the Harmsen laboratory

(described previously by Dr. R. Coffman of DNAX Research Inst. Palo Alto, CA [213,214]) and suspended in 500µl sterile PBS.

Lipophilic Dye-labeling of Resident Cells

Mice were labeled with lipophilic dye via i.v. injection of DiD or $DiIC_{18}(5)$ Vybrant Cell-labeling dye (Molecular Probes, Eugene OR) suspended at a 10 fold dilution from stock in a 2% BSA carrier to a final volume of 100µl per injection. Prior to any subsequent experimental procedures, mice were rested for one week to ensure that cells newly recruited to the site of infection would be dye low to dye negative.

Flow Cytometery and
Analysis of Cellular Phenotypes

BALF samples were spun at 210G for 8 minutes in a Sorval benchtop RT7 centrifuge. Cell pellets were resuspended in 100µl Fc block to prevent non-specific antibody binding and incubated for 10 minutes on ice, subsequently samples underwent a 30-minute dark incubation with commercially available anti-mouse CD11b, CD11c, SiglecF, Ly6c, and Ly6G fluorophore-conjugated antibodies (BD Biosciences, BD Pharmingen, San Diego, CA). After a rinse step, samples were examined on FACSCantos, LSR II, or Acuri flow cytometers (Becton Dickinson, Mountain View, CA). Unstained cells from lavage samples collected at the highest infective dose used for each experiment were run to control for autofluorescence. Further data analysis and graph creation was performed with FlowJo software (Ashland, OR).

Histology of Cytospin Preparations
With Visualization by Microscopy

Aliquots (100μl) were taken from resuspensions of FACS solutions fixed in ice cold EtOH for one hour in the dark and adhered to a slide via cytospin centrifugation (Shandon Cytospin). Cell surface staining for CD11c was achieved using a FITC rat anti-mouse CD11c antibody (BD Biosciences, BD Pharmingen, San Diego, CA). Bacteria were visualized either by fluorescent protein expression (GFP NMII) or by permanent red staining with *C. burnetii* as the target antigen (Dako North America, Inc., Carpinteria, CA). Slides were coverslipped with ProLong Gold antifade reagent with DAPI (Molecular Probes/Invitrogen, Eugene OR) and imaged at 40X on a Nikon E800 fluorescent scope (Nikon Instruments, Melville NY). Composite image overlays were made using NIS-Elements Nikon Image Analysis software.

Cell Counts, Differentials and Total Cell Analysis

Total cells counts were obtained for BALF on a standard hemocytometer, using trypan blue exclusion of dead cells. Cytopsins stained with Diff-Quick (Siemens Healthcare Diagnostics, Newark DE) for differential analysis allowed for enumeration of various cell-types. Total cells for each individual type were obtained by multiplying total cell counts by the percentage for each individual type.

Statistical Analysis

Prism Software (Prism Software Corporation, Orange County, CA) was used to produce all graphs and perform statistical analysis on data. All data (excluding FACS plots and photomicrographs) is expressed as log transform of change in bacterial burden.

Graphs show mean and standard error of the mean (SEM) for all groups plotted. Student's T test was run to test for statistically significant differences between paired groups, $p<0.01$ was considered significant. ANOVA was run to test for statistically significant differences between multiple groups, $p<0.05$ was considered significant.

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CHAPTER THREE

THE FACULTATIVE INTRACELLULAR PATHOGEN CRYPTOCOCCUS NEOFORMANS PROLIFERATES PRIMARILY EXTRACELLULARLY EARLY IN IN VIVO INFECTION

Contributions of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper

Senior Co-author: Robert A. Cramer

Contributions: Conceived and designed the experiments, critically read and edited the manuscript

Senior Co-author: Allen G. Harmsen

Contributions: Conceived and designed the experiments, contributed reagents/materials/analysis tools, critically read and edited the manuscript

Manuscript Information Page

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mBio

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____ Published in a peer-reviewed journal

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Abstract

Recent studies support the ability of *Cryptococcus neoformans* to replicate intracellularly,particularly in alveolar macrophages (AM), leading to classification as a facultative intracellular pathogen. However, it is not known the extent to which intracellular replication, as opposed to extracellular replication of *C. neoformans*, contributes to *C. neoformans* growth in the lungs. Here we utilized a GFP expressing strain of *C. neoformans* and further labeled these organisms with CM-DiI before infection. Because GFP expression remained relatively constant with or without *C. neoformans* replication, but the CM-DiI staining waned upon replication, whether individual yeast had replicated *in vivo* could be assessed by FACS analysis. At different times after infection, *C. neoformans* were isolated from either purified alveolar macrophages (intracellular) or the cell-free fraction of lavage fluids (extracellular) and analyzed for GFP and CM-DiI fluorescence. We found that in the lungs, a limited number of *C. neoformans* underwent limited replication intracellularly in AM, while the majority of *C. neoformans* that replicated *in vivo* did so extracellularly and to a greater extent than intracellular yeast. In fact, the majority of intracellular fungal load in AM at 48 hrs post-infection was attributable to the uptake of extracellular *C. neoformans* from 24 to 48 hrs post-infection and was not a result of intracellular replication.

Importance

Cryptococcus neoformans is a human pathogen capable of causing primary pulmonary infection. Following primary infection, *C. neoformans* may spread

systemically within the host and infiltrate the central nervous system, often causing severe and chronic cryptococcosis. *C. neoformans* is a facultative intracellular pathogen, able to replicate both extracellularly and within host macrophages. In fact, it has been hypothesized that host macrophages may facilitate systemic spread of the fungus. We show here that while *C. neoformans* is capable of intracellular replication within the host macrophage *in vivo,* extracellular replication is the predominant determinant of fungal burdens early in pulmonary infection. To our knowledge, this is the first study to directly compare intracellular and extracellular replication during early *in vivo* pulmonary infection. Our findings indicate that if the host macrophage does provide a conducive niche for fungal infection, it is subsequent to the first 48 hrs of infection.

Introduction

The genus *Cryptococcus* is classified as an encapsulated yeast comprised of two species, *Cryptococcus neoformans* and *Cryptococcus gattii* [10]. Both cryptococcal species can be found in natural environmental samples from wood or tree sources, as well as, bird droppings [215] and both species are capable of infecting human hosts, most often causing self-limiting pulmonary infection with instances of both chronic pulmonary infection and central nervous system infiltration being reported [10]. *C. gattii* has gained recent notoriety in news reports as the causative agent of potentially lethal cryptococcosis outbreaks in immunocompetent individuals throughout Northwestern North America [216]. *C. neoformans* has long been recognized as a potential human pathogen; typically thought to be a risk only to immunocompromised individuals, *C. neoformans* is now

being recognized as a pathogenic risk for immunocompetent individuals as well [217]. However, with recent global cryptococcal meniningitis cases among HIV infected individuals being estimated at nearly 1 million individuals per year, cryptococcal infection still represents a serious health risk for the immunocompromised [14].

C. neoformans has been shown to be susceptible to phagocytosis and killing by neutrophils, macrophages and dendritic cells [218,219]. In particular, species-specific differences in AM function within rats and mice have been implicated in the observed differences in cryptococcosis susceptibility between these two species [220]. From this, it was concluded that the enhanced anti-cryptococcal ability of rat AM accounted for the observed decreased susceptibility to cryptococcosis when compared to mice. This would seem to mark the alveolar macrophage as a pivotal cell in the determination of the course and outcome of infection.

Consistent with this, *Cryptococcus* has been shown to posses several means to counter-act the host immune response [168,221]. This interplay between yeast and host innate immunity is not straightforward. In fact, the presence of neutrophils, in particular at the site of infection, has been shown to worsen clinical symptoms of infection in a murine model of infection [222].

Moreover, despite the ability of phagocytes to kill *C. neoformans*, research supports a role for monocytes in the systemic spread of fungal infection within the host in a murine model of infection [172]. Indeed, *C. neoformans* has been shown to undergo *in vitro* replication within human macrophages [223]. These findings have been recapitulated and supported by *in vivo* work within a murine model of infection and have

resulted in *C. neoformans* being redefined as a facultative intracellular pathogen [15].

Cryptococcus is also known to be able to escape the macrophage through an active process of phagosomal expulsion [184,185,224]. It has been shown that Th1 and Th17 type responses control cryptococcal infection and drive increased rates of macrophage expulsion of the fungus. Whereas, Th2 type immune responses decrease the rates of fungal expulsion and increase rates of intracellular replication resulting in a worsening of infection [101]. Additionally, there has been speculation that the AM may function as a permissive niche perpetuating cryptococcal infection under certain conditions [171]. It has also been suggested that whether initiation of infection results from inhalation of spores or yeast forms of *Cryptococcus* will directly affect the clearance or persistence of infection [225].

Thus, fungal interaction with the host immune system is varied and complex. The course of this interaction early in infection is likely to affect the progression and outcome of the infection in the long-term. Despite the role AM have been suggested to play in supporting intracellular replication of *C. neoformans*, the extent to which this intracellular replication contributes to *C. neoformans* burdens as compared to extracellular replication, is not known.

We employed a novel strategy for dye-labeling yeast that allowed both the identification of infected cells and the determination of *C. neoformans* replication. We show that within the first 48 hrs of infection, AM were permissive to limited intracellular *C. neoformans* replication. However, at 48 hrs post-infection, the majority of *C. neoformans* cells contained within AM had been extracellular within the previous

24 hrs. Extracellular replication, within the first 48 hrs of infection, far exceeded intracellular replication and was sufficient to account for the majority of the observed increase in fungal burden. Thus, despite being a facultative intracellular pathogen, we show it is extracellular replication that serves as the key determinant of fungal burden early in *in vivo* infection.

Results

C. neoformans Replication in Liquid Culture Results in a Decrease in the Dye Intensity of Lipophilic Dye Labeled Yeast

To determine whether lipophilic dye labeling provided a reliable method for tracking yeast replication, H99 *C. neoformans* was incubated with the lipophilic dye CM-DiI. Liquid cultures were then seeded with dye labeled *C. neoformans* and dye intensity of individual yeast was determined at 2,4,6 and 8 hrs of growth in media. Total numbers of *C. neoformans* were determined by hemocytometer counts. CM-DiI intensity was determined by flow cytometery at each time-point. *C. neoformans* numbers increased 2.64 times in the first 8 hrs of liquid culture, as determined by hemocytometer counts (Figure 3-1A). This marked a statistically significant increase in yeast numbers ($p<0.05$ 8 hour culture vs. all other conditions by ANOVA). Mean fluorescence intensity for CM-DiI dye labeling exhibited about a halving across this same timecourse (Figure 3-1B-representative histogram). Dye intensity exhibited a bimodal distribution on yeast in culture for 8 hrs. This suggested that not all *C. neoformans* had undergone division by this time. These results indicated that CM-DiI labeling provided a reliable method for

assessing the degree to which a *C. neoformans* population has undergone replication in liquid culture. Additionally, CM-DiI labeling provided a method for identifying those individual yeast that have undergone replication.

Wild-type H99 *C. neoformans* and Ras-GFP H99 *C. neoformans* Exhibit No Significant Differences in Liquid Culture Growth or Fungal Burdens Isolated From Infected Animals

To allow both for an enhanced fungal tracking and identification method and a baseline measure against which to gauge dye dilution marked fungal replication, we wished to take advantage of a GFP-expressing mutant strain of H99 *C. neoformans* in subsequent experiments. This H99 derived strain contains an ectopically integrated GFP tagged Ras construct that results in high GFP signals [226]. To rule out potential pleiotropic effects in this strain, it was first necessary to characterize fungal growth in liquid media, as well as, the resultant fungal burdens in infected mice, to ensure that no significant growth and survival defects occurred because of the introduced Ras-GFP construct. Wild-type H99 *C. neoformans* and Ras-GFP H99 *C. neoformans* showed similar growth in YPD liquid media across 24 hrs, when assessed by hemocytometer fungal counts (Figure 3-2A).

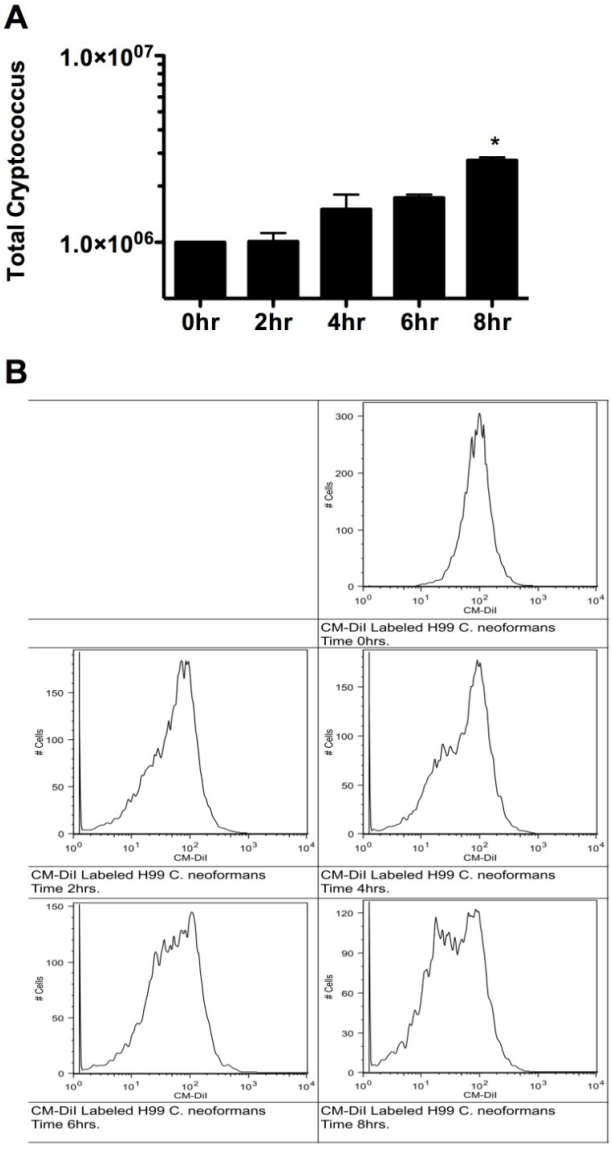


Figure 3-1- Growth of CM-DiI labeled C. neoformans in YPD liquid media resulted in a loss of dye intensity commensurate with an increase in yeast numbers.
CM-DiI labeled wild-type H99 *C. neoformans* was grown in YPD liquid media. CM-DiI fluorescence intensity and *C. neoformans* numbers were determined at 0,2,4,6 and 8 hrs of growth. A) CM-DiI labeled wild-type H99 *C. neoformans* exhibited an approximately 2.5 fold increase in total numbers from 0 to 8 hrs in YPD liquid media. No growth defects were observed as a result of CM-DiI labeling. B) CM-DiI dye fluorescence intensity exhibited a nearly one-log decrease across approximately half the total cell population in culture. The overall *C. neoformans* population exhibited a bimodal distribution of CM-DiI intensity at 8 hrs in YPD liquid media. ($p<0.05$ 8 hrs vs. all other times; ANOVA)

There were no significant differences in final fungal numbers between the two groups (p=0.1323). Next, BALB/c mice were inoculated with $1X10^6$ of either wild-type H99 *C. neoformans* or Ras-GFP H99 *C. neoformans* and lung burdens of the fungi determined 48 hr later. Mice hemocytometer counts of lavage fluid samples collected from infected animals (Figure 3-2B). There were no significant differences in the observed fungal burdens between the two groups of mice (p=0.1173). Taken together, these data suggest that no significant *in vitro* or *in vivo* growth defects exist between H99 and GFP-Ras H99 *C. neoformans* strains.

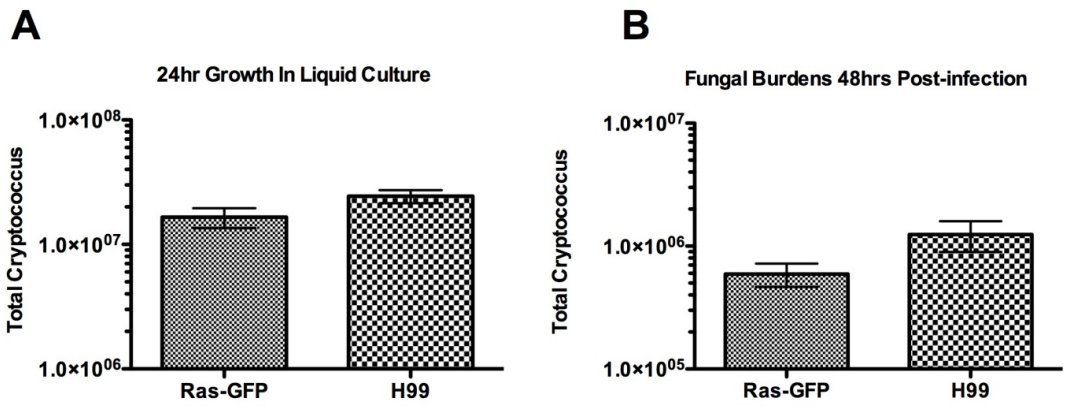


Figure 3-2- GFP expression in Ras-GFP H99 C. neoformans does not alter fungal growth in liquid media or fungal burdens in infected animals.

A) $2X10^6$ Ras-GFP H99 *C. neoformans* or wild-type H99 *C. neoformans* was grown in YPD media for 24 hrs. Hemocytometer fungal counts were performed and no significant difference in fungal growth was observed. B) BALB/c mice were inoculated with either $1X10^6$ Ras-GFP H99 *C. neoformans* or wild-type H99 *C. neoformans*. 48 hrs post-inoculation, bronchoalveolar lavage fluid was collected and hemocytometer fungal counts were performed. No significant differences in fungal burdens were observed between groups.

C. neoformans Replication in Cell Culture Results in a Decrease in the Dye Intensity of Lipophilic Dye Labeled Yeast

To determine whether the diminishment of dye intensity represented a reliable indication of *C. neoformans* replication in cell culture, as was observed in liquid culture, J774 murine macrophages were inoculated with CM-DiI labeled Ras-GFP H99 *C. neoformans*. At this time, cultures were observed to contain both intracellular and extracellular *C. neoformans* populations. Total numbers of *C. neoformans* were determined by hemocytometer counts following collection of culture supernatants along with scraping and water lysis of J774 monolayers. Total *C. neoformans* numbers increased 2.5 times from 2 to 24 hrs in cell culture (Figure 3-3A). This marked a statistically significant increase in yeast numbers ($p=0.0007$ by Student's T test). Mean fluorescence intensity for CM-DiI dye labeling exhibited over a five-fold reduction across this same timecourse (Figure 3-3B- representative histogram). By 24 hrs in cell culture, *C. neoformans* dye intensity exhibited a single population peak. This suggested that nearly all *C. neoformans* had undergone division at this time, or that non-replicating cells had been digested. These results indicated that CM-DiI labeling provided a reliable method for assessing the degree to which a *C. neoformans* population had undergone replication in cell culture under mixed intracellular and extracellular conditions.

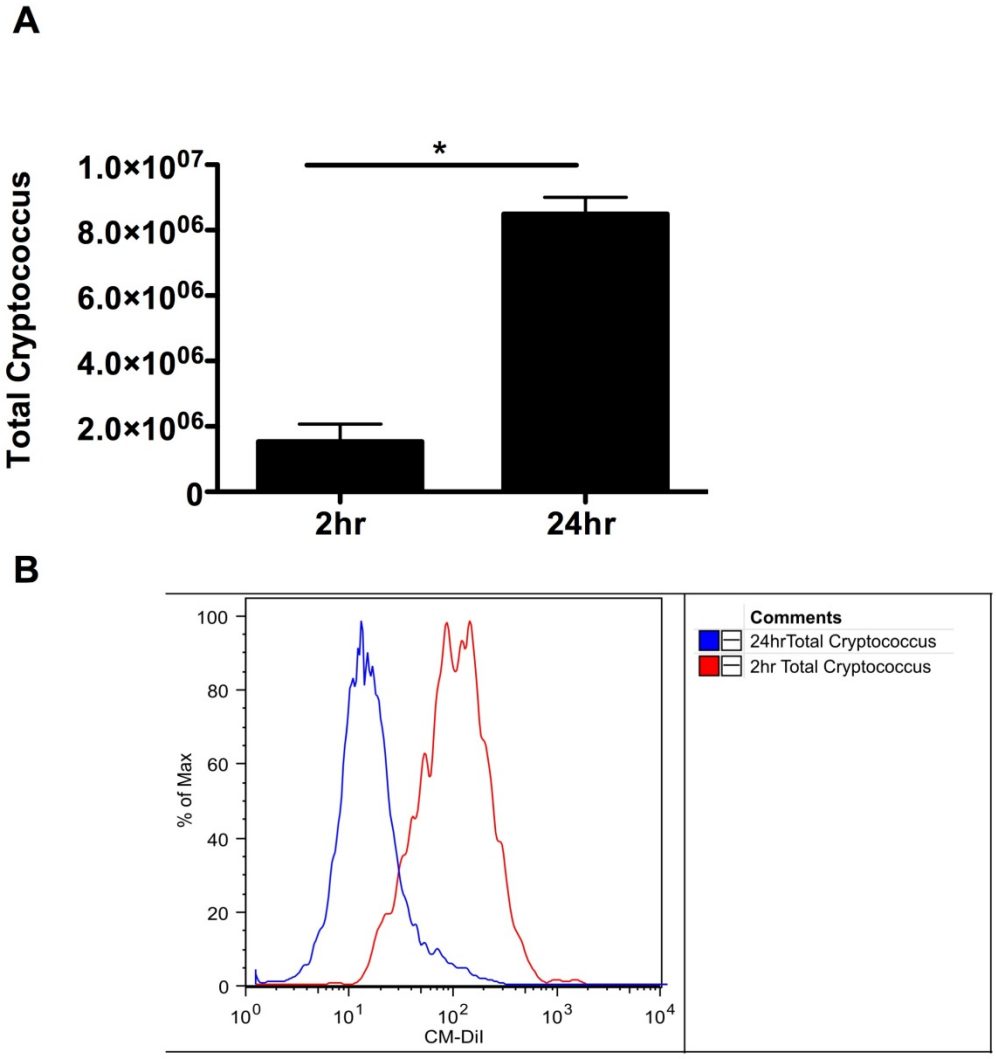


Figure 3-3- Growth of CM-DiI labeled C. neoformans in J774 cell culture resulted in a loss of dye intensity commensurate with an increase in yeast numbers.

CM-DiI labeled Ras-GFP H99 *C. neoformans* was grown in co-culture with J774 cells. CM-DiI fluorescence intensity and *C. neoformans* numbers were determined at 2 and 24 hrs of growth. A) CM-DiI labeled Ras-GFP H99 *C. neoformans* exhibited greater than a two-fold increase in total numbers from 2 to 24 hrs in co-culture with J774 cells. No growth defects were observed as a result of CM-DiI labeling. B) CM-DiI dye fluorescence intensity exhibited a one-log decrease in average fluorescent intensity across the 22 hr co-culture. (p=0.0007; Student's T-test)

C. neoformans Replication *in vivo*
Results in a Decrease in the Dye
Intensity of Lipophilic Dye Labeled Yeast

To determine whether the diminishment of dye intensity represented a reliable indication of *C. neoformans* replication *in vivo*, BALB/c mice were inoculated with either $1X10^6$ viable or heat killed CM-DiI labeled Ras-GFP H99 *C. neoformans*. Bronchoalveolar lavage fluid (BALF) samples were collected and subjected to yeast cell counts and FACS analysis. *C. neoformans* recovered from animals having received viable yeast exhibited a 3-fold increase in yeast numbers by hemocytometer count (data not shown) between 4 and 24 hrs post-inoculation. By FACS analysis non-cell associated GFP^+ *C. neoformans* exhibited a commensurate 5-fold decrease in mean fluorescence intensity of CM-DiI across the same time-course (Figure 3-4A). No similar decrease in mean fluorescence intensity of CM-DiI was observed for heat killed non-cell associated *C. neoformans* (Figure 3-4B). These results indicated that loss of CM-DiI labeling over time provided a reliable method for assessing the degree to which individual *C. neoformans* had undergone replication *in vivo*.

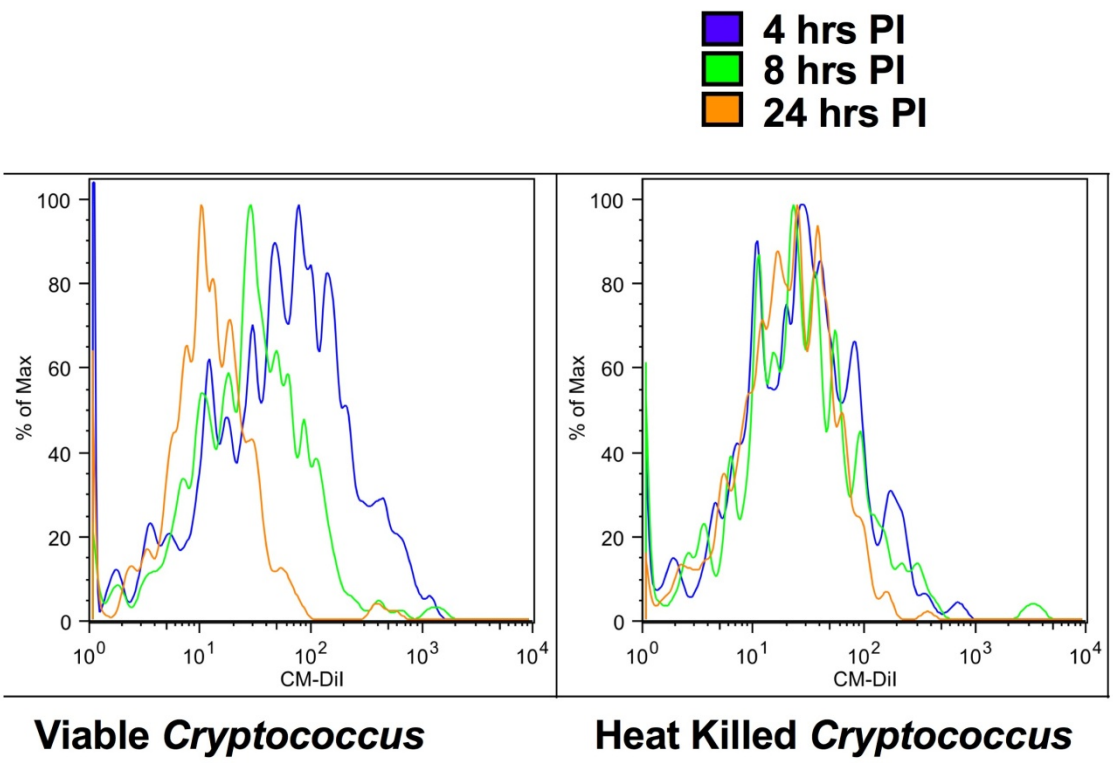


Figure 3-4- Live, but not heat killed, C. neoformans exhibited a decrease in CM-DiI intensity in the lungs of BALB/c mice.

A) BALB/c mice were inoculated with $1X10^6$ viable CM-DiI labeled Ras-GFP H99 *C. neoformans*. From 4 hrs post-inoculation to 24 hrs post-inoculation yeast recovered in bronchoalveolar lavage fluid exhibited a progressive reduction in CM-DiI fluorescent intensity. B) BALB/c mice were inoculated with $1X10^6$ heat-killed CM-DiI labeled Ras-GFP H99 *C. neoformans*. From 4 hrs post-inoculation to 24 hrs post-inoculation yeast recovered in bronchoalveolar lavage fluid did not exhibit any reduction in CM-DiI fluorescent intensity.

C. neoformans Replication *in vivo* Results in an
Increase in Total *C. neoformans* Numbers
Predominated by an Increase in Extracellular *C. neoformans*

To determine the contributions of either extracellular or intracellular replication to total *C. neoformans* numbers during infection, BALB/c mice were inoculated with $1X10^6$ viable CM-DiI labeled Ras-GFP H99 *C. neoformans*. At 4, 24 or 48 hrs post-inoculation, BALF was collected and run on a $CD11c^+$ MACS selection column. Resultant populations of column flow-through or $CD11c^+$ column retained cells were collected. Microscopic observations of the two column populations confirmed that the retained fraction consisted of AM, many with intracellular *C. neoformans*, and that the column flow through fraction contained limited numbers of neutrophils and only extracellular *C. neoformans*. In separate experiments purified AM that had phagocytized *C. neoformans* *in vivo* were stained with Calcoflour, which does not stain intracellular *C. neoformans*. Results indicated that the *C. neoformans*, isolated from the AM, had not been stained with the Calcoflour indicating that the yeast were indeed intracellular and not just adherent to the AM. Intracellular and extracellular samples were subjected to water lysis. Determination of CM-DiI labeling intensity of GFP^+CD45^- *C. neoformans* revealed a progressive decrease in dye intensity in the extracellular population. This decrease was similar irrespective of prior water lysis, indicating that water lysis did not alter the fluorescent dye intensity. At 48 hrs post-inoculation, the extracellular population was indistinguishable from an unlabeled negative control sample (Figure 3-5A and 3-5B). This was consistent with extracellular fungal replication during the time-course of infection. In contrast, while the intracellular population did exhibit some diminishment in

CM-DiI, approximately 50% of the intracellular population had not lost staining intensity and was indistinguishable from the dye labeled inoculum (Figure 3-5C). This was consistent with limited intracellular replication. Representative fungal counts by FACS showed a significant increase in extracellular, but not intracellular, fungal numbers from 24 to 48 hrs post-inoculation (Figure 3-5D). This was consistent with the FACS data and supported extracellular fungal replication as the driving force behind overall increases in fungal burden within the first 48 hrs of infection. Additionally, in some of the mice, a pulse of Calcoflour was administered via intra-tracheal inoculation at 24 hrs post-inoculation. This was done to label the *C. neoformans* extracellular at that time. At 48 hrs post-inoculation nearly all the extracellular *C. neoformans* were Calcoflour labeled and interestingly, about three-quarters of the intracellular *C. neoformans* were also Calcoflour labeled. This indicated that most of the *C. neoformans* that were intracellular at 48 hrs had been extracellular at 24 hrs and thus were phagocytized during that period (Figure 3-5E). This suggests that much of the apparent intracellular replication seen in Figure 3-5C may actually have occurred extracellurarly before the *C. neoformans* were phagocytized. In addition, that nearly all the extracellular *C. neoformans* at 48 hrs were Calcoflour labeled indicates that very few of those yeast were not extracellular 24 hrs previously. Thus, it seems unlikely that many of the extracellular yeast at 48 hrs could have come from an intracellular source between 24 and 48 hrs. Taken together, these results strongly suggest that the replicating pool of *C. neoformans* resides primarily in the extracellular compartment in the lungs early in infection.

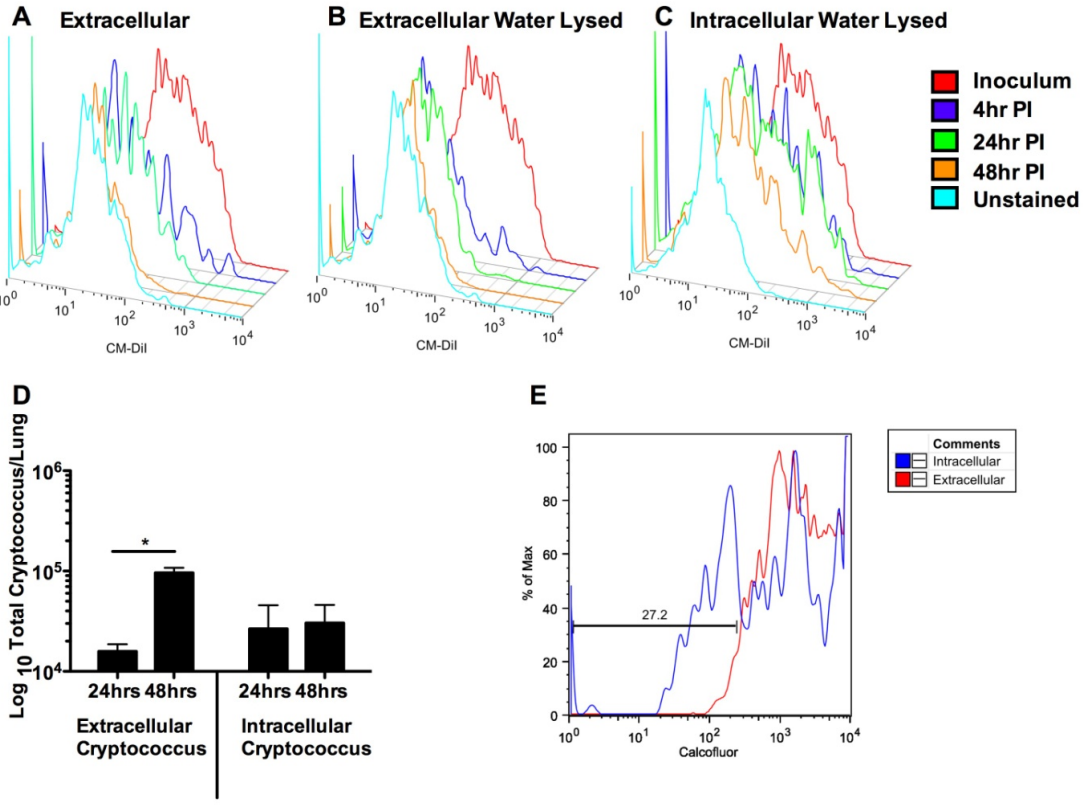


Figure 3-5- Between 4 and 48 hrs post-infection, extracellular but not intracellular C. neoformans exhibited significant replication in the lungs of BALB/c mice.

BALB/c mice were inoculated with $1X10^6$ viable CM-DiI labeled Ras-GFP H99 *C. neoformans*. At 4 hrs post-inoculation, 24 hrs post-inoculation and 48 hrs post-inoculation, bronchoalveolar lavage fluid was collected and pooled into three populations: extracellular, having passed through a CD11c MACS selection column; extracellular water lysed, having passed through a CD11c MACS selection column and having been subsequently water lysed; intracellular, having been retained on a CD11c MACS selection column and having been subsequently water lysed. A) A progressive reduction in dye intensity, consistent with extracellular fungal replication, was observed throughout the infection. B) Water lysis had no effect on the progressive reduction in dye intensity; the observed results are comparable to those of the extracellular non-lysed pool and consistent with no influence on replication by neutorphils. C) Minor reduction in dye intensity was observed on intracellular fungus through the course of infection. This is consistent with limited intracellular fungal replication. D) Fungal counts were performed by FACS and showed significant fungal replication only for extracellular *C. neoformans.* These results are consistent with those observed by FACS analysis of dye intensity. E) Intratracheal inoculation at 24 hrs post-infection of Calcoflour and subsequent comparison of extracellular and intracellular fungus revealed that 73% of the intracellular fungus and nearly all of the extracellular fungus had been extracellular at 24 hrs post-infection. (p=0.02; Student's T-test)

From 2 to 48 hrs Post-infection the Number of
CM-DiI positive AM Increased While the Percentage
of AM Harboring Intact *C. neoformans* Decreased

The FACS analysis described above determined the amount of replication (CM-DiI intensity) of individual, intact (by forward and side scatter), *C. neoformans* (GFP^+CD45^-). However, FACS analysis of lavage samples could not distinguish whether CM-DiI^+ AM contained intact *C. neoformans* (as would happen with intracellular replication) or had phagocytized and digested the yeast and had recycled the CM-DiI into their own lipids (as would happen with AM killing of the *C. neoformans*). Thus, fluorescent microscopy was performed on cells in lavage samples from Ras-GFP H99 *C. neoformans* inoculated mice at 2, 24, and 48 hrs post-inoculation. Intact fungus was visually determined based on the presence of large, round, intracellular spherules consistent with fungal morphology observed in inoculum samples. It was found that the number of CM-DiI positive macrophages increased (Figure 3-6A and 3-6B) during this time. This finding was consistent with the uptake of previously extracellular fungus accounting for the majority of intracellular fungus at 48 hrs post-infection. However, at 2 hrs most CM-DiI^+ AM contained intact yeast (Figure 3-6C- representative example); whereas, by 48 hrs, fewer CM-DiI^+ AM contained visibly intact yeast (Figure 3-6D-representative example). Quantification of the percentage of CM-DiI^+ AM that contained morphologically intact *C. neoformans* is shown in Figure 3-6E and determined by counting the number of CM-DiI^+ AM containing intact yeast per 100 CM-DiI^+ AM in each of five animals per experimental timepoint. The results indicate that, over time, the percentage of CM-DiI^+ AM that contain morphologically intact *C. neoformans* decreases.

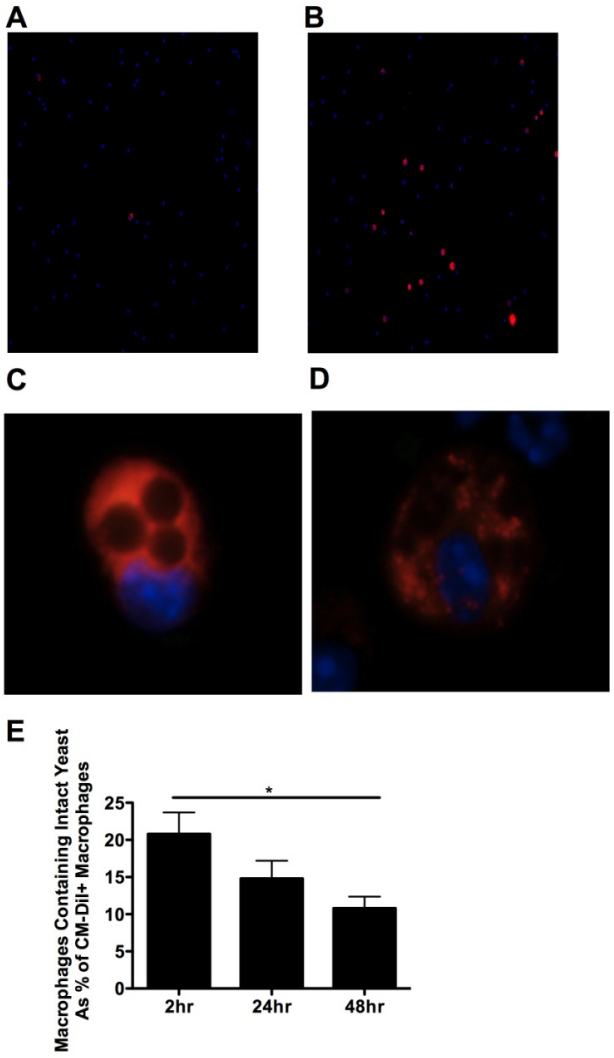


Figure 3-6- While the number of CM-DiI positive alveolar macrophages increased between 2 and 48 hrs post-infection, the percentage of macrophages containing viable yeast decreased.

BALB/c mice were inoculated with $1X10^6$ viable CM-DiI labeled Ras-GFP H99 *C. neoformans*. From 2 hrs post-inoculation to 48 hrs post-inoculation bronchoalveolar lavage fluid was collected and cytospins were performed. A) 2 hrs post-infection, CM-DiI^+ cells were visualized at 10X magnification B) 48 hrs post-infection, CM-DiI^+ cells were visualized at 10X magnification. An increase in CM-DiI^+ cells is clearly visible at 48 hrs, relative to the 2 hr timepoint. C) A representative CM-DiI^+ macrophage is shown with intact *C. neoformans* D) A representative CM-DiI^+ macrophage is shown without intact *C. neoformans* E) The percentage of CM-DiI^+ macrophages was determined through the course of infection by a visual anlaysis of five different samples per timepoint. A significant reduction in CM-DiI^+ macrophages containing intact yeast was observed from 2 to 48 hrs post-inoculation. ($p<0.05$; ANOVA)

This result is not consistent with significant *C. neoformans* replication occurring in the AM.

Depletion of Alveolar Macrophages Did Not Significantly Alter *C. neoformans* Burden in the Lungs of Infected Mice

If indeed, extracellular, as opposed to intracellular, replication of *C. neoformans* was the predominant determinant of *C. neoformans* burden, we would expect that depleting AM from the lungs would have little effect on *C. neoformans* replication in the lungs. Thus, wild-type C57Bl/6 or Mafia mice were treated with dimerizing agent to facilitate AM depletion in the susceptible Mafia animals (AM depletion in these animals occurs through dimerizing agent induced macrophage specific Fas-mediated apoptosis [227]). Subsequent to depletion, all animals were inoculated with $1X10^6$ viable Ras-GFP H99 *C. neoformans*. At 24 hrs post-inoculation, BALF was collected and total *C. neoformans* burdens were determined by FACS. This depletion strategy significantly depleted BALF $CD11c^+$, which were predominantly AM (Figure 3-7A). Indeed, depletion of $CD11c^+$ AM during the first 24 hrs of infection did not result in a significant decrease in *C. neoformans* burdens, compared to non-depleted wild-type animals (Figure 3-7B). These data strongly suggest that during the first 24 hrs of infection, intracellular replication of *C. neoformans* in AM is not a major contributor to growth of *C. neoformans* in the lungs.

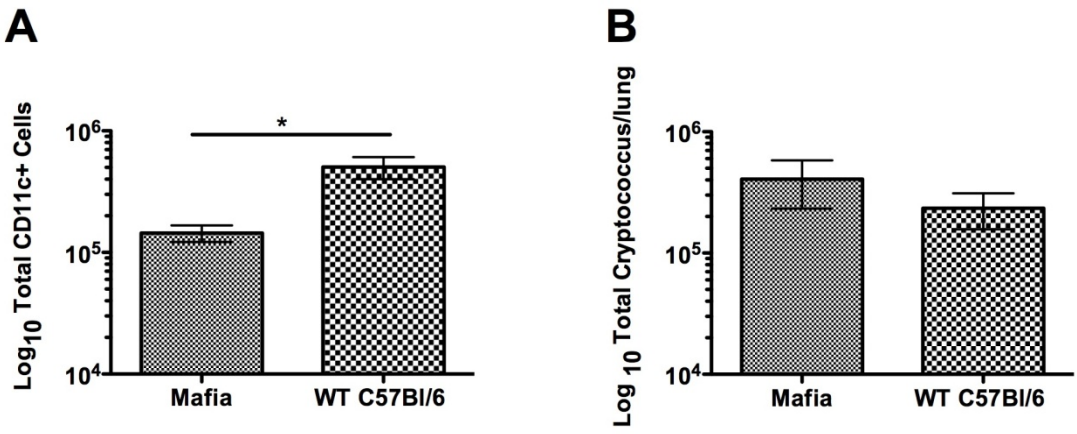


Figure 3-7- Depletion of alveolar macrophages did not result in a significant change in fungal burden.

Mafia mice and wild-type C57Bl/6 mice were treated with dimerizing agent in two doses prior to infection. Both groups were then infected with $1X10^6$ CM-DiI labeled Ras-GFP H99 *C. neoformans* for 24 hrs. A) Dimerizing agent mediated depletion resulted in a significant decrease in the number of $CD11c^+$ cells within the lungs of Mafia mice compared to wild-type controls. B) Dimerizing agent mediated depletion did not result in a significant difference in the *C. neoformans* burden within the lungs of Mafia mice compared to wild-type controls. (p=0.008; Student's T-test)

Discussion

Here we have demonstrated a lipophilic dye labeling strategy allowing for the identification of individual *C. neoformans* having undergone replication. We have employed this dye labeling strategy to provide what is, to the best of our knowledge, the first study to directly compare the relative contribution of intracellular and extracellular *C. neoformans* replication on yeast burden early in the context of lung infection. We show that whereas intracellular replication did occur to some extent, extracellular replication was the predominate means by which *C. neoformans* burdens increased early during lung infection. Indeed, depletion studies confirmed that AM had relatively little

bearing on overall fungal burdens, through either facilitating replication or clearance, in the first 24 hrs of infection.

The current study does not contradict the literature supporting the classification of *C. neoformans* as a facultative intracellular pathogen [15,223,228]. In fact, we present data here supporting the idea that *C. neoformans* is capable of limited replication within the host AM. The ability of different *C. neoformans* strains to replicate intracellularly has been tied to observed strain dependent virulence differences. The H99 *C. neoformans* strain appears to exhibit both an intermediate capacity for intracellular replication and intermediate virulence [229]. However, we show here that, extracellular replication was the dominant factor driving the observed increase in early fungal burdens. Certainly, we cannot exclude the possibility that some *C. neoformans* is capable of persisting in AM. This leaves open the possibility that certain subsets within the heterogeneous lung macrophage population present at the site of pulmonary infection may function to shelter *C. neoformans* and contribute to chronic infection. Currently within our lab, this is an area of ongoing study. However, the data presented here argues strongly that if such a susceptible niche does exist it is extremely restricted and likely not to be readily apparent within the first 48 hrs of infection.

To our knowledge, the data presented here are the first to address, in the context of fungal replication and total fungal burden, the possible contribution to lung yeast burden by *C. neoformans* non-lytically escaping the macrophage after intracellular replication [230]. Our data indicates that *C. neoformans* intracellular replication is minimal and that possible subsequent escape from AM in a non-lytic fashion does not

significantly contribute to the burden of *C. neoformans* in the lungs early in infection. This was determined by our result of not seeing a significant Calcoflour negative population in the extracellular *C. neoformans* population in our "pulse label experiment" (Figure 3-5E). In this regard, had yeast replicated within AM and non-lytically escaped AM between 24 and 48 hrs they would not have been Calcoflour positive at 48 hrs since they would have been intracellular at the time of Calcoflour deposition in the lungs. Without this additional assessment, it is quite possible that the microscopically observed increase in dye positive macrophages (Figure 3-6A and 3-6B) from 2 to 48 hrs post-infection could have been interpreted as intracellular replication. However, it must be emphasized that our experiments were limited to the first 48 hrs of infection because of the complete waning of DiI staining after that time. Thus, we cannot rule out that after 48 hrs of infection, intracellular replication increases and non-lytic escape of yeast from AM then contributes significantly to yeast burdens.

It has been shown that depletion of lung resident AM and dendritic cells in CD11c DTR mice results in death by six days post-infection with *C. neoformans* [231]. Interestingly, the authors of this study found that while depletion of dendritic cells and alveolar macrophages was lethal, there was no observed increase in fungal burden resulting from depletion. Instead, there was an observed increase in neutrophils and B cells in the lungs of CD11c DTR mice. From this, the authors concluded that the key role for dendritic cells and alveolar macrophages is not in fungal clearance; instead, they suggest, macrophages serve to alter the inflammatory environment preventing the induction of a deleterious early immune response.

While we did not observe a significant difference in fungal burdens in our Mafia mouse model, it is possible that AM are capable of effecting some degree of fungal clearance. This clearance may be masked by the inoculum dose, the time of assessment and the efficacy of our depletion strategy. However, some clearance of the *C. neoformans* by AM in the first 48 hrs of infection is consistent with our microscopy data (Figure 3-5A and 3-5B).

Here we present data that supports extracellular replication as the predominant determinant in the progression of early pulmonary *C. neoformans* infection. Whereas it is possible that AM could play a pivotal role in the outcome of *C. neoformans* burden (supporting yeast intracellular replication or clearance) in a more protracted infection, the data presented here argues strongly that this role was not evident during the first 48 hrs of infection. Indeed, our findings indicated that extracellular replication of *C. neoformans* was the predominate driving factor in the establishment of early pulmonary *C. neoformans* infection.

Materials and Methods

Ethics Statement

All experimental procedures performed were in accordance with Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee at Montana State University, Bozeman, MT (Protocol permit # 2012-09). All efforts were taken to minimize pain and suffering.

Cryptococcus Strains

Wild-type *Cryptococcus neoformans* strain H99 was a gift from Dr. Connie Nichols in the laboratory of Dr. Andrew Alspaugh at Duke University Medical Center. This strain was cultured and maintained on YPD agar media (Becton Dickinson, Cockeysville, MD). Liquid YPD was inoculated and cultures were grown on a New Brunswick Scientific shaker-table incubator (New Brunswick Scientific, Edison, NJ) at 37°C and 125RPM for 48 hours prior to infection.

Ras-GFP H99 *Cryptococcus neoformans* strain H99 mutant was a gift from Dr. Connie Nichols in the laboratory of Dr. Andrew Alspaugh at Duke University Medical Center [226]. This strain was cultured and maintained identically to the wild-type. Heat killed *C. neoformans* was prepared by a 1 hour incubation at 60°C.

C. neoformans Growth and
Collection in Cell Culture

J774 murine macrophages (ATCC J774A.1 ATCC® TIB-67™) were grown in T-175 culture flasks to a confluent monolayer with DMEM media supplemented with 10% FBS and 1% Pen/Strep. Growth was maintained in a cabinet incubator at 37°C with 5% CO_2. At experimental timepoints, the monolayer was scraped and collected with the culture media. Cells and media were centrifuged at 210G for 8 minutes in a Sorval benchtop RT7 centrifuge. Pellets were resuspended in 5 ml of water and vortexed vigorously and continuously for five-minutes. Samples were again centrifuged at 210G for 8 minutes in a Sorval benchtop RT7 centrifuge. Pellets were resuspended in 100µl of FcR block and examined on an LSR II (Becton Dickinson, Mountain View, CA).

Animals

As noted, groups of five six to eight week old age and sex matched BALB/c (commercially available and raised in-house at Montana State University), C57Bl/6 (commercially available and raised in-house at Montana State University), or Mafia (commercially available at Jackson Labs, Bar Harbor, Maine strain name: C57BL/6-Tg(Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6)2Bck/J) mice were used in all experiments.

Intratracheal Infection of Mice

In all experiments, mice were lightly anaesthetized with isoflurane and inoculated intratracheally (i.t.) with final concentrations of *C. neoformans* suspended in 100μl sterile phosphate-buffered saline (PBS).

Collection of Bronchoalveolar Lavage

Bronchoalveolar lavage fluid (BALF) was collected as previously described [91]. Mice were given an intraperitoneal (i.p.) injection of a Phenobarbital solution and exsanguinated. Following this, mice were cannulated via a small tracheal incision; the lungs were flushed with aliquots of Hanks' balanced salt solution with 3 mM/liter EDTA in a final volume of 5ml.

Histology of Cytospin Preparations
With Visualization by Microscopy

Aliquots (100μl) were taken from resuspensions of FACS and adhered to a slide via cytospin centrifugation (Shandon Cytospin). CM-DiI labeled *C. neoformans* was visualized at either 2 or 48 hours post-infection. Slides were coverslipped with ProLong

Gold antifade reagent with DAPI (Molecular Probes/Invitrogen, Eugene OR) and imaged at either 10X or 40X as noted on a Nikon E800 fluorescent scope (Nikon Instruments, Melville NY). Composite image overlays were made using NIS-Elements Nikon Image Analysis software.

Column Selection and Preparation of
Intracellular and Extracellular *C. neoformans*

BALF samples were subjected to sorting and cell selection using LS selection columns and CD11c magnetic beads as per manufacturer's protocols (Miltenyi Biotec Inc., Auburn, CA). Subsequent to column selection, both column flow-through and $CD11c^+$ samples were water lysed and processed for flow cytometeric analysis as described below.

Flow Cytometery and Analysis of Cellular Phenotypes

BALF samples were centrifuged at 210G for 8 minutes in a Sorval benchtop RT7 centrifuge. Pellets were resuspended in 100μl FcR block to prevent non-specific antibody binding and incubated for 10 minutes on ice. Water lysis was performed on specified samples by resuspending pellets in 5 ml of water and vortexed vigorously and continuously for 5 minutes. Subsequently samples underwent a 30-minute dark incubation with commercially available anti-mouse CD45 (clone 30-F11) and CD11c (cloneN418) fluorophore-conjugated antibodies (BD Biosciences, BD Pharmingen, San Diego, CA) along with 100 μl of 0.05% Calcofluor White (Becton, Dickinson and Company, Sparks, MD). After a rinse step, 100 μl of a known concentration of BD Calibrite APC beads (BD Biosciences, San Jose, CA) was added and samples were

examined on an LSR II (Becton Dickinson, Mountain View, CA). Further data analysis and graph creation was performed with FlowJo software (Ashland, OR).

Determination of *C. neoformans*
Burden by Flow Cytometery

For the purposes of flow cytometery, *C. neoformans* was defined as $CD45^-$, Calcofuor White $^+$, GFP^+. Beads were identified by forward/side scatter and were APC^+. Total numbers of *C. neoformans* per sample were determined by flow cytometery using the following formula: Total *C. neoformans* per sample = *C. neoformans* per gate (Total beads per sample/ Beads per gate).

Lipophilic Dye-labeling of *C. neoformans*

C. neoformans was labeled with CM-DiI (Invitrogen Life Technologies, Grand Island NY) by suspending $1x10^6$ *C. neoformans* per milliliter in a 1:10 dilution of CM-DiI from a stock concentration of 1 mg/ml for one half-hour on a New Brunswick Scientific shaker-table incubator at 37°C and 125RPM. Samples were then stored overnight at 4°C. Prior to experiments, samples were washed twice in sterile DPBS.

Cell Counts, Differentials and Total Cell Analysis

For all experiments, total cells counts were obtained for BALF on a standard hemocytometer, using trypan blue exclusion of dead cells. Cytopsins were prepared and stained with Diff-Quick (Siemens Healthcare Diagnostics, Newark DE) for differential analysis with enumeration of various cell-types and total cells for each individual type obtained by multiplying total cell counts by the percentage for each individual type.

Determination of *C. neoformans* Burden by Hemocytometer

For all experiments, total cells counts were obtained for BALF on a standard hemocytometer, using trypan blue exclusion of dead cells. Notation was made, where appropriate, for both total extracellular and total intracellular *C. neoformans.*

Statistical Analysis

Prism Software (Prism Software Corporation, Orange County, CA) was used to produce all graphs and perform statistical analysis on data. All data (excluding FACS plots and photomicrographs) is expressed as total numbers of cells. Graphs show mean and standard error of the mean (SEM) for all groups plotted. Student's T test was run to test for statistically significant differences between paired groups, $p<0.01$ was considered significant. ANOVA was run to test for statistically significant differences between multiple groups, $p<0.05$ was considered significant.

Acknowledgements

The authors wish to thank Dr. Connie Nichols in the laboratory of Dr. Andrew Alspaugh at Duke University Medical Center for the kind donation of H99 *Cryptococcus neoformans* and Ras-GFP H99 *Cryptococcus neoformans*. The authors also wish to thank the members of the Harmsen Laboratory for technical assistance in performing the experiments presented herein. This work was supported by NIH/COBRE GRANT# P20GM103500 and INBRE-BRIN GRANT# P20GM103474. Additional Support was provided by The Montana State University Agricultural Experiment Station and The MJ Murdock Charitable Trust.

CONCLUSIONS AND FUTURE STUDIES

Despite a complex and tightly integrated network of physical barriers and innate immune defenses within the mammalian lung, the lung is still susceptible to infection. In general, invading pathogens either evade the host immune network or subvert some part of the host network in order to facilitate survival. We hypothesized that because of their location, control by immune suppression, and functional role in orchestrating the pulmonary defense network, resident AMs would be a cell population susceptible to infection. We further hypothesized that once infected, AM would be permissive to the maintenance of intracellular pathogens, due to the tightly regulated activation state of the AMs serving to prevent hyper-inflammatory responses to pathogenic insult. We tested these hypotheses in the context of pulmonary infection with either the obligate intracellular bacteria *C. burnetii* or the facultative intracellular fungus *C. neoformans*.

In chapters 2 and 3 of this dissertation, we show that the AM population responds to pulmonary infection caused by either *C. burnetii* or *C. neoformans* in a pathogen-dependent manner. Whereas both pathogens are capable of intracellular replication in AMs, the resident AM population provides a niche for *C. burnetii* replication and decidedly does not provide a niche for significant *C. neoformans* replication early in pulmonary infection. Indeed, in the context of *C. neoformans* infection, it is the extracellular environment of the lung that permits the majority of early fungal replication.

During infection with *C. burnetii*, the resident AM provides both a susceptible and permissive niche for the maintenance of infection. Consistent with this, lower infective doses of the bacteria result in greater bacterial replication, as compared with

high infective doses of the bacteria. In presenting these data, we rule out the possibility that higher infective doses of bacteria result in greater systemic dissemination within the host, or that these higher doses result in an increased severity of infection. Moreover, we also rule out a possibility that increased inflammation, resulting from higher infective doses of bacteria, is sufficient to drive enhanced bacterial clearance during a high dose infection. Our work supports, as an explanation for this observation, the theory that higher infective doses quickly fill the conducive cellular niche of the resident AMs, with the remaining bacteria being forced into less hospitable environments. Importantly, we also show a time- and dose-dependent phenotypic change in resident AMs marked by the upregulation of surface integrin CD11b expression, combined with maintained expression of surface integrin CD11c. Lastly, we show that it is the resident AM and not the newly recruited monocyte which is susceptible to infection.

During infection with *C. neoformans*, we show that within the first 48 hrs of infection, the majority of observed fungal replication takes place in the extracellular environment. We demonstrated that while occurring, intracellular replication within the AMs, is only a minor contributor to the observed increases in fungal burden in the host. On the contrary, the majority of intracellular fungus isolated at 48 hrs post-infection had been extracellular within the intervening 24 hrs. Thus, the observed intracellular burden was attributable not to replication but to phagocytosis of extracellular fungi. When AMs were depleted we saw no statistically significant difference in fungal burdens at 48 hrs post-infection, as compared with the burdens in non-depleted animals. This result is

inconsistent with the AM providing a niche for fungal survival and replication early during pulmonary infection.

Having identified AMs as being susceptible to infection by *C. burnetii*, but not *C. neoformans*, it is of interest to indentify the mechanistic causes for this observed difference. Moreover, it is of interest to determine the mechanistic cause for the continued permissiveness of AMs to *C. burnetii* infection. In the context of *C. burnetii* infection, we observed the importance of $CD11b^{+}/CD11c^{+}$ resident AMs as a susceptible and permissive cell type. As was discussed in the introduction, this phenotype is consistent with AM activation and may also be exhibited by recruited monocytes entering the lung during pulmonary inflammation [80]. This is consistent with the data we present in chapter 2 of this dissertation. While beyond the scope of the experiments presented in chapter 3, it would be interesting to determine whether this $CD11b^{+}/CD11c^{+}$ resident AM population was similarly observed during pulmonary infection with the fungal pathogen or if this population was not present until the recruitment of inflammatory monocytes in response to the infection. It is possible that *C. neoformans* leaves the AM population largely unactivated. This would be consistent with our findings that *C. neoformans* appears to avoid early interactions with AMs and that AMs do not provide a niche for early fungal replication. Instead, it is the extracellular environment where the majority of early fungal replication occurs and it is quite possible that effective immune responses are not truly initiated until the recruitment of monocytes to the site of infection has occurred. Indeed, a role for recruited monocytes in the successful clearance of *C. neoformans* has been recognized [232,233]. As observed by Duan et. al., the

$CD11b^{+}/CD11c^{+}$ recruited monocytes simultaneously exhibited markers of M1 and M2 activation [80]. Determining whether the dual M1/M2 activation also occurs in the context of *C. burnetii* or *C. neoformans* infections would be very informative. Additionally, it would be of interest to determine whether this observed mixed activation state would lead to either a Th1- or Th2-type adaptive immune response. A Th1-type mediated adaptive immune response is known to be critical in the clearance of both *C. burnetii* both *C. neoformans* [92,94]. It is quite possible that the activation, recruitment and subsequent return to homeostasis occurring in the context of either *C. burnetii* or *C. neoformans* infection is responsible for setting the necessary environmental pattern to allow for a productive adaptive immune response. If this were true, we may expect to see differences in the responses of either the resident AMs or recruited monocytes during infection. Likewise, such an observation would open the possibility that induced alterations in either the resident AM population or the recruited monocyte population could skew the adaptive immune response to infection.

Moving forward with future experiments to investigate these possibilities, the following working hypothesis could be adopted: activation and subsequent resolution of activation within either the resident AM or the recruited monocyte populations follows differing, but potentially converging pathways dictated by the pulmonary environment. Ultimately, these environmental effects determine the efficacy of subsequent adaptive immune responses in the clearance of pulmonary pathogens.

Unpublished observations in the Harmsen laboratory with IL-10 knock-out and integrin $\alpha_v\beta_6$ knock-out mice have shown no significant differences in susceptibility to *C.*

burnetii infection when compared to wild-type mice. This could indicate that, contrary to the literature, TGF-β and IL-10 do not play an important role in the control of pulmonary *C. burnetii* infection. Alternatively, these observations could indicate that these effectors play a context-dependent role in the control of pulmonary *C. burnetii* infection. This would be more consistent with the information presented in the introduction, where the coordinated and interrelated network of pulmonary immune mechanisms exhibit a high degree of overlap and context dependence.

We have shown here that AM susceptibility and permissiveness to infection varies between pulmonary infection with *C. burnetii* and *C. neoformans*. In the context of the former, resident AMs are susceptible to and permissive of infection and a majority of infected resident AMs exhibit a characteristic phenotype with possible functional implications. In the context of pulmonary *C. neoformans* infection, the resident AM does not provide a susceptible or permissive niche for the establishment of early pulmonary infection. In fact, the resident AM may function to facilitate minor clearance of *C. neoformans*. During early infection, the extracellular environment of the lung appears to be the susceptible and permissive niche for *C. neoformans* growth. These findings fill deficiencies in the literature and provide an *in vivo* analysis of both susceptibility and permissiveness within select pulmonary environments during early infection with either an obligate or a facultative intracellular pulmonary pathogen. More importantly, the findings presented in chapters 2 and 3 of this dissertation, set the stage for a mechanistic investigation of interactions between innate and adaptive pulmonary immune mechanisms. These future investigations should yield detailed information about what

kind of immune responses lead to resolution or to chronic infection during pulmonary infections with *C. burnetii* or *C. neoformans* infection.

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